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Origins and Consequences of Altered Metabolic Processes in Obese Pregnant Women

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Abstract

Maternal obesity is an increasing concern in the obstetric population. It confers increased morbidity and mortality to the mother and offspring during pregnancy and delivery as well as potential long-term increase in risk of ill health to the offspring. There are currently few effective interventions and no pharmacological therapies. Potential mechanisms to account for ill health in obese non-pregnant individuals include excess inflammation, both systemically and within specific tissues such as adipose, as well as alterations in metabolic regulation including hyperglycaemia, reduced sensitivity to insulin and altered adipokine expression. In healthy pregnancy, there are significant adaptations to maternal metabolism, including the development of profound systemic insulin resistance. We hypothesize that there exists an interaction between the metabolic adaptations of pregnancy and those occurring in obesity which could provide a physiologically plausible mechanism which could contribute to the pathogenesis of adverse outcomes associated with obese pregnancies. In this thesis, we sought to understand and define the metabolic adaptations to pregnancy in severely obese women. Anthropometric characteristics are described in a longitudinal case-control study of apparently healthy obese ($\text{BMI} > 40\text{kg/m}^2$) pregnant women. Systemic adipokine and pro-inflammatory cytokine profiles were measured using ELISA. Indices of insulin sensitivity were assessed at three time points in pregnancy. In a cohort study of healthy pregnant women in the third trimester, transcript levels of adipokines and inflammatory cytokines in paired subcutaneous and omental adipose tissue biopsies were quantified and correlated these transcript levels with booking body mass index (BMI). Obese pregnant women gained less weight in pregnancy compared to lean women, but had significantly elevated fasting third trimester glucose, as well

as elevated blood pressure and fasting insulin resistance throughout pregnancy. Fasting leptin was elevated throughout pregnancy in obese compared with lean pregnancy women; however, in the third trimester there was no correlation between adipose tissue leptin mRNA levels and BMI. Transcript levels of IL-6 were positively correlated with BMI in subcutaneous but not omental adipose tissue; no other positive correlations with BMI were shown.

Hyperinsulinaemic euglycaemic clamps with concomitant use of stable isotope tracers were carried out in a case-control study of healthy obese pregnant women to characterise in detail whole body insulin sensitivity, endogenous glucose production and rate of lipolysis. In contrast to the original hypothesis, by the third trimester, there were few differences between lean and obese pregnant women in whole body glucose disposal (WGD) and endogenous glucose production. Compared with non-pregnant women, lean pregnant women demonstrated approximately 60% decrement in WGD; in contrast, obese non-pregnant women were already significantly insulin resistant but did not develop further insulin resistance in response to pregnancy.

3-Tesla (3T) Magnetic Resonance Imaging (MRI) and ¹H-Magnetic Resonance Spectroscopy (¹H-MRS) was used to assess abdominal fat distribution, hepatic and skeletal muscle lipid content in a case-control study of healthy pregnant women in the third trimester. As expected, obese pregnant women have greater adipose accumulation in both subcutaneous and intra-abdominal adipose depots and greater lipid accumulation in skeletal muscle. However, hepatic lipid content was low in both groups and there were no significant differences between lean and obese pregnant women. This was not expected as both groups are profoundly insulin resistant at this at this gestation, and

in non-pregnant individuals, insulin resistance at this level would be expected to drive hepatic lipid accumulation, and may point to a pregnancy-specific hepato-protective mechanism.

In conclusion, in this thesis, it has been shown that while obese women are insulin resistant with an adverse metabolic profile, that there does not appear to be the expected worsening of this profile in response to pregnancy and that by the end of pregnancy, lean women have a similar phenotype. Instead, while lean women are exposed to this environment only towards the end of pregnancy, obese women and their offspring are exposed throughout gestation, including key periods of fetal development in early pregnancy. This prolonged exposure may account for the excess pathologies in such pregnancies, potentially by exhausting what physiological reserve such women have pre-pregnancy. Potential therapies must therefore be optimally timed to improve the metabolic profile of obese women in early pregnancy, without hindering the required adaptations of the third trimester.

Declaration

Except where acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. The work described in this thesis has not been previously accepted for, or is currently being submitted in candidature for another degree.

Chapter 3

I acknowledge the assistance of Norma Forson, Zillah Jones, Ellie Golightly, Rose Leask, Graham Harold, Nanette Hibbert and the Edinburgh Reproductive Tissue Biobank team for assistance in collection and processing of the blood and tissue samples. Norma Forson, Zillah Jones and myself carried out glucose tolerance testing of participants; Graham Harold carried out the insulin and NEFA assays.

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'If we knew what it was we were doing, it would not be called research, would it?'

Attributed to Albert Einstein

Presentations and Publications Relating to this Thesis

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- 'Adipose Tissue Metabolism in Obese Pregnant Women'
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- 'Pro-inflammatory gene mRNA levels are elevated in subcutaneous but not visceral adipose tissue in obese pregnant women'
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Medical Research Society Clinician Scientists. London, February 2011
- 'The Effect Of Obesity in Pregnancy On Relative Expression of Adipokines In Human Subcutaneous and Omental Adipose Depots.'
Sarah M Barr, Nicholas M Morton, Brian R Walker and Jane E Norman
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- 'Assessment of intra-hepatic and intra-muscular lipid in obese pregnant women: an application of 3-tesla Magnetic Resonance Imaging'
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Publications

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- F C Denison, K A Roberts, S M Barr and J E Norman. Obesity, pregnancy, inflammation, and vascular function. *Reproduction*. 2010 Sep;140(3):373-85.

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Abbreviations

11 β -HSD1	11-beta dehydroxysteroid dehydrogenase 1
17 β -HSD2	17-beta hydroxysteroid dehydrogenase 2
ADA	American Diabetes Association
ADIPR	Adiponectin receptor
ADP	Air Displacement Plethysmography
AMPK	AMP-activated protein kinase
ANOVA	Analysis of Variance
ATGL	Adipose Triglyceride Lipase
AU	Arbitrary Units
BAT	Brown Adipose Tissue
BIA	Bioelectrical Impedance Assessment
BMI	Body Mass Index
BSA	Body Surface Area
cAMP	cyclic adenosine monophosphate
CMACE	Centre for Maternal and Child Enquiries
CRIC	Clinical Research Imaging Centre
CRP	C-Reactive Protein
CSF	cerebrospinal fluid
CT	Computed Tomography
Ct	Cycle threshold
CV	coefficient of variation
DAG	Diacylglycerol
DBP	Diastolic blood pressure
DEXA	Dual Energy Xray Absorptiometry
DGAT2	Diglycerol Acyl Transferase 2
DHEA-S	dehydroepiandrosterone sulphate
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
EGIR	European Group for the Study of Insulin Resistance
EGP	Endogenous glucose production
ELISA	Enzyme-linked Immunosorbant Assay
ERTBB	Edinburgh Reproductive Tissue BioBank
FABP	Fatty Acid Binding Protein
FASN	Fatty Acid Synthase
FATP	fatty acid transport protein

FFA	Free fatty acids
FFM	Fat-free Mass
FLASH	Fast Low Angle SHot
FM	Fat Mass
FSIVGTT	Frequently Sampled IntraVenous Glucose Tolerance Test
GC	Glucocorticoid
GC-MS	Gas Chromatography Mass Spectrometry
GDM	Gestational Diabetes Mellitus
GIR	Glucose Infusion Rate
GLUT1	GLUcose Transporter 1
HASTE	HAIf fourier Single shot Turbo spin Echo
HDL	High-Density Lipoprotein
HEC	Hyperinsulinaemic Euglycaemic Clamp
hGH	Human Growth Hormone
HOMA - IR	HOmeostasis Model Assessment - Insulin Resistance
hPGH	Human Placental Growth Hormone
hPL	Human Placental Lactogen
HPLC	High-performance liquid chromatography
HSL	Hormone Sensitive Lipase
ICD	International Classification of Disease
IDF	International Diabetes Federation
IGF1	Insulin-like Growth Factor 1
IL	Interleukin
Ins-R	Insulin receptor
IR	Insulin Resistance
IRS1	insulin-receptor substrate-1
IS	Insulin Sensitivity
ISI	Insulin Sensitivity Index
IUGR	Intrauterine Growth Restriction
LDL	Low-Density Lipoprotein
LEPR	Leptin receptor
LPL	Lipoprotein Lipase
LREC	Lothian Research Ethics Committee
MAPK	Mitogen-activated Protein Kinase
MAPK	mitogen-activated protein kinase

MCP1	Monocyte Chemoattractant Protein 1
ME1	Malic Enzyme 1
MHO	metabolically healthy obese
MONW	metabolically obese but normal weight
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
MRS	Magnetic Resonance Spectroscopy
NEFA	Non-esterified Fatty Acid
NHS	National Health Service
NICE	National Institute for Health and Clinical Excellence
NPY	neuropeptide-Y
OGTT	Oral Glucose Tolerance Test
Om	Omental
PAI1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PI3K	phosphoinositide-3-kinase
PKA	Protein Kinase A
PKA	protein kinase A
PKB	Protein Kinase B
PKB	protein kinase B/Akt
PKC θ	Protein Kinase C theta
POMC	pro-opiomelanocortin
PPAR alpha	Peroxisome Proliferator-activated Receptor alpha
PPAR γ	Peroxisome Proliferator-activated Receptor gamma
PRESS	Point Resolved multi Echo Single shot Spectroscopy
PRL	Prolactin
QUICKI	Quantitative Insulin-Sensitivity Check Index
RBP4	Retinol Binding Protein
RBP4	Retinol binding protein 4
ROS	reactive oxygen species
SBP	Systolic blood pressure
Sc	Subcutaneous
SCD1	Stearoyl-CoA desaturase 1
SD	Standard deviation
SEM	Standard error of the mean

SIGN	Scottish Intercollegiate Guidelines Network
SPD	Symphysis Pubis Dysfunction
TAG	Triacylglycerol/Triacylglyceride
TAMRA	Carboxytetramethylrhodamine
TBW	Total Body Water
TMB	3,3',5,5'-tetramethylbenzidine
TNF α	Tumour Necrosis Factor Alpha
UWW	Underwater weighing
VEGF	vascular endothelial growth factor
VIBE	Volumetric Interpolated Breathhold Examination
Visc	Visceral
VLDL	Very Low-Density Lipoprotein
WAT	White Adipose Tissue
WGD	Whole-body glucose disposal
WHO	World Health Organisation
WHR	Waist-Hip Ratio

Chapter 1 Introduction

Obesity is a disorder of excess adipose tissue, and in pregnancy, it is associated with a number of adverse short- and long-term outcomes for both mother and offspring. The incidence of obesity is increasing in the UK, in parallel with the global situation; in particular, the long term impact of the maternal metabolic environment on offspring outcomes such as obesity and cardiovascular disease in later life is just beginning to be recognised, contributing to a huge burden of disease for the future.

The therapeutic interventions available for obese pregnant women and their care providers are currently limited by three broad factors: firstly by an incomplete understanding of the function and regulation of adipose tissue in health, this area having undergone a significant paradigm shift in the last two decades; secondly, by a rapidly evolving appreciation of the effects of obesity on adipose tissue function; and thirdly, by our limited understanding of maternal metabolism in normal pregnancies and the complex interactions of the fetomaternal unit. While it is clear that interventions are required to prevent propagation of ill health to subsequent generations, the combined complexities of the combination of obesity and pregnancy are therefore a significant challenge.

This thesis will examine the role of adipose tissue in the metabolic adaptations of pregnancy in obese women. Firstly, methods employed to assess body fat content and distribution, as well as functional aspects of obesity including assessment of insulin sensitivity are reviewed. Pregnancy impacts not only the practicalities of employing some of these techniques

which are widely used in the general population but also their applicability. In this thesis, several complementary methods of assessment of adipose tissue accretion and distribution and of insulin sensitivity were employed to address these issues. Secondly, the normal structure and function of adipose tissue in health is reviewed, focussing on the regulation of lipolysis, the endocrine function of adipose tissue and followed by coverage of the evidence for the disruption of adipose tissue homeostasis by obesity in non-pregnant individuals. In this thesis, the metabolic and adipose tissue characteristics of severely obese pregnant women are described and contrasted with those of healthy weight pregnant women. Thirdly, the normal adaptation of lipid metabolism and its regulation by insulin by pregnancy is addressed. Lastly, the impact of maternal obesity in pregnancy is summarised, along with the consequences for the fetus.

1.1. Obesity

1.1.1. Incidence & impact in pregnancy

Obesity is defined as 'abnormal or excessive fat accumulation that may impair health' (World Health Organisation (WHO), 2000). The International Classification of Disease (ICD-10) code E66 includes several subgroups including morbid obesity (E66.8) and extreme obesity with alveolar hypoventilation (Pickwickian syndrome, E66.2)

The incidence of obesity is increasing worldwide, although there is some evidence to suggest that incidence may be reaching a plateau, more so among women than men (Flegal, Carroll *et al.* 2010). In the UK, it was estimated that approximately 24% of men and women were obese (BMI>30kg/m²) in 2007 (WHO). In Scotland in 2008, it was estimated that approximately 66% of men and 59% of women aged 18-65 were obese. In the obstetric population, the incidence of obesity (BMI>30kg/m²) has increased from 7.6% to 15.6% in England from 1989 to 2007, with greatest increases in the percentage of women with morbid obesity (BMI>40kg/m²) (Heslehurst, Rankin, *et al.* 2010). In Scotland, the incidence of women with booking BMI >30kg/m² was estimated at approximately 20% in 2005 (Kanagalingam, Forouhi, *et al.* 2005).

Pre-gravid obesity is associated with adverse outcomes for mother and neonate, including maternal and fetal mortality (Cnattingius, Bergstrom, *et al.* 1998; Castro, Avino, *et al.* 2002; Jensen, Ovesen, *et al.* 2005; Denison, Price, *et al.* 2008). Women who are obese have reduced fertility, and in the event of conception, have an increased risk of miscarriage (Lashen, Fear, *et al.* 2004).

They are at increased risk of the major morbidities of pregnancy: gestational diabetes, pre-eclampsia, obstetric cholestasis and venous thromboembolism as well as increased mortality, largely due to thromboembolic events (CMACE, 2005-2008).

Obese women are also at increased risk of minor complications of pregnancy including chest infection, symphysis pubis dysfunction (SPD), oesophageal reflux, and carpal tunnel syndrome (Denison, Norrie, *et al.* 2009). The excess financial cost to the NHS is high.

At the end of pregnancy, obese women are at increased risk of post-dates pregnancy (Denison, Price, *et al.* 2008); approximately one third of women with BMI>35 undergo induction of labour in the UK (CMACE), compared with one fifth of the general population. This is associated with an elevated risk of late stillbirth. Operative or instrumental delivery is also increased, almost 40% of women with BMI>35 deliver by caesarean section compared to 25% in the general population. This is important because operative deliveries are associated with increased morbidity including haemorrhage, anaesthetic complications, and post-partum complications including thromboembolism and wound infections. Anaesthetic complications include difficulty in siting regional analgesia, increased requirement for general anaesthesia and increased risk of Mendelson syndrome.

Fetal issues include increase risk of congenital anomaly (Rasmussen, Chu, *et al.* 2008; Stothard, Tennant, *et al.* 2009; Mills, Troendle, *et al.* 2010). This is possibly due to a true 'metabolic' teratogenesis similar to that observed in diabetic pregnancies (Suhonen, Hiilesmaa, *et al.* 2000) and nutritional defects such as folate deficiency (Kaidar-Person, Person, *et al.* 2008), as well as decreased sensitivity of antenatal screening tests such as ultrasound (Dashe,

McIntire, *et al.* 2009). Fetal loss is increased, both first trimester miscarriage and late stillbirth. Neonatal admission rates are also increased (Sarkar, *et al.* 2007), as is the likelihood of pre-term delivery (Smith, Shah, *et al.* 2007; McDonald, Han, *et al.* 2010). There can also be difficulties with achieving acceptable fetal monitoring in labour; a low threshold for use of a fetal scalp electrode is recommended by the Royal College of Obstetricians & Gynaecologists (CMACE/RCOG, 2010).

More importantly, the impact of the maternal metabolic environment on long-term offspring outcome is beginning to be recognised, with increased risk of obesity in later life in the offspring of obese mothers (Whitaker, 2004; Oken, Taveras, *et al.*, 2007.). However, these relationships are complex and are affected by postnatal environmental factors.

1.1.2. Assessing Body Fat content and distribution

The most commonly used method to quantify 'fatness' is the Body Mass Index (BMI). This is a means to describe weight relative to height, is expressed in kg/m², and is calculated as follows:

$$\text{BMI} = \text{weight (kg)} / [\text{height (m)}]^2$$

The World Health Organisation defines BMI as follows:

Category	BMI Range (kg/m ²)
Underweight	<19.9
Normal	20.0 – 24.9
Overweight	25.0 – 29.9
Obese Class I	30.0 – 34.9
Obese Class II	35.0 – 39.9
Obese Class III (morbid obesity)	>40.0

BMI has the advantage of being easily and reproducibly measured, simply calculated and universally accepted, thus facilitating comparison between data from different countries. However, there are a few caveats which should be considered. The first is international variation on the basis of ethnicity. For example, South East Asian and Japanese populations use different ranges to categorise BMI: BMI > 23.0 is considered overweight in these populations as there is evidence that this ethnic group have elevated cardiovascular risk even if BMI is low compared to those of European ethnicity (Razak, Anand, *et al.* 2007). Secondly, as BMI is a simplistic estimation of total body fatness, the assumption that relative proportions of muscle, water and fat mass remain constant leads to overestimation of adiposity in individuals with increased lean body mass e.g. athletes. Therefore while in population based

studies BMI is a useful comparator, in more detailed studies, greater description of body composition is required. Thirdly, it is increasingly evident that it is not simply total body fat which determines risk of cardiovascular disease, but body fat distribution, particularly excess central or abdominal adiposity (Kannel, Cupples, *et al*, 1991). Thus additional assessment of body composition and distribution is required. There are many approaches to this which can be broadly classified as either direct or indirect methods. Indirect methods infer body composition; reference methods involve direct measurement from which body composition, commonly expressed as percentage body fat, can be calculated. Indirect methods include body mass index, anthropometric measurements such as waist/hip ratio and skinfold thicknesses, and use of bioimpedance to estimate body composition using electrical conductivity. Direct methods involve more complex techniques such as underwater weighing (UWW) or air-displacement plethysmography (ADP), Dual Energy X-ray Absorptiometry (DEXA) and stable isotope tracer methods. All of these approaches require certain assumptions to be made and application of a generalisable model of body composition, the simplest of which is the 2-compartment model, which splits the organism into Fat Mass (FM) and Fat Free Mass (FFM). Fat free mass is most commonly estimated by assessment of total body water, using the assumption that fat is relatively anhydrous. The second assumption that it uses is that FFM is composed of 73% water, 20% protein and 7% bone mineral. Models of body composition can be employed; the simplest is the 2-compartment model, which divides the body into FM and FFM; a 4-compartment model divides FFM into water, bone mass and muscle mass. The choice of applied model depends on the question of interest, size and nature of the study as well as resources available.

1.1.2.1. Indirect Methods

Anthropometric measurements

The simplest measurement of body distribution uses the ratio of waist circumference to hip circumference, the waist-hip ratio (WHR). A high WHR (>0.85) has been associated with increased central distribution of adipose tissue, which in turn has been associated with increased risk of cardiovascular disease and type II diabetes mellitus (Kannel, Cupples, *et al.*, 1991).

A more detailed assessment of body fat distribution uses measurement of skinfold thickness using callipers at specific anatomical locations, commonly including triceps, biceps, subscapular and supra-iliac regions. These measurements can be used to calculate the total body fat as a percentage of total body weight. There are a number of different equations for calculating body fat; commonly used are the Jackson-Pollock 3- and 7-site equations (Jackson, Pollock, *et al.*, 1980) and Durnin/Womersley (Durnin & Womersley, 1974) equations and their variations. The advantages of these types of measurements are that they are cheap to use, accessible and acceptable to patients, which makes them very useful for 'field' studies of large populations. The major drawback is that obtaining the measurements can be extremely operator dependent, both in identifying the correct site for measurement and in use of the callipers to obtain measurements. While intra-observer variability can be minimised with training and repetition, there is significant variability in estimation of total body fat depending on the choice of equation used to calculate body fat. There is also an additional source of variability where the gender and ethnicity of the population being measured differs from the characteristics of the study cohort used to develop

those equations, or where body fat content is at the extremes of the Gaussian distribution i.e. extremely obese or extremely lean. For example, some studies have shown a consistent 3-8% overestimate of total body fat when anthropometric measurements were compared with underwater weighing in a group of females with predominantly upper body fat distribution (Swan & McConnell, 1999). Additionally, skinfold derived estimations of body fat can be inaccurate compared with other methods. However, while assessment of absolute body fat may be inaccurate, skinfold measurements can be used to assess longitudinal changes in body composition. In pregnancy, while calliper measurements are highly correlated with ultrasound measurement of subcutaneous adipose depth, they also consistently overestimate the true skinfold thickness compared to ultrasound. This is thought to be due to altered compressibility of subcutaneous tissue in pregnancy (Stevens-Simon, Thureen, *et al.* 2001).

Bioelectrical Impedance

Bioelectrical impedance analysis (BIA) makes use of measurement of the degree of impedance of tissues to conductivity of an electrical current to calculate the relative water content of a living organism and thus the relative proportion of fat and fat free mass can be subsequently estimated. Bioimpedance measurements have been validated against other Total Body Water (TBW) approaches, and correlate closely in the studied populations (Kushner & Schoeller, 1986). In addition, the equipment required is easily used and portable. However, BIA can be unreliable in conditions where generalisable equations for the derivation of TBW have not been validated or hydration status is not known. In pregnancy, the contribution of amniotic fluid volume and altered total body water is variable, although some small

studies have suggested that BIA is comparable to isotopic dilution methods (Lukaski, Siders., *et al* 1994).

1.1.2.2. Direct Methods

Total body water

Assessment of total body water (TBW) can be used to infer fat mass by the application of the 2-compartment model. There are a number of methods for measuring total body water: isotopic dilution methods and volume displacement techniques. Isotopic dilution methods make use of stable isotope tracers such as deuterium (^2H , or D) and oxygen -18 (^{18}O); deuterated water (D_2O) can be used alone to calculate total body water, or doubly labelled water (D_2^{18}O) can be used to calculate metabolic rates. This method works by introducing a sample of labelled molecule, e.g. deuterated water, of known concentration into a system (eg an organism) and then measuring how much that sample has been diluted in the total population of water molecules present in that system (Schoeller, Santen, *et al.* 1980). The total mass of water is therefore calculated as follows:

$$\text{Nmole} = \text{Mmole} / \text{pM}$$

Where Nmole is the total number of water molecules, Mmole is the number of molecules introduced and pM is the proportion of marked molecules in the sample obtained after a period of time.

These methods are straightforward to carry out, but can be expensive and require access to mass spectrometry equipment to analyse samples, which limits their usefulness for larger studies.

Water displacement (underwater or hydrostatic weighing) and air displacement methods rely on calculation of body density to estimate fat content, by making a precise measurement of weight and body volume based on volume or pressure change. This data can then be used to calculate total body density and thus estimate % body fat based on the equations of Siri (Siri, 1961):

$$\% \text{Fat} = 495 / \text{density} - 450$$

These methods are more accurate than simple anthropometric measurements, have good agreement with other methods of assessing body composition and have been accepted as the gold standard method of assessing body composition. However, they require access to specialised equipment and training, are time-consuming to carry out, and are therefore less suitable for large population studies. Air displacement plethysmography (ADP) is generally more acceptable to patients than underwater weighing and can be useful for assessment of specific subgroups of patients such as infants, in whom other methods of assessing body composition are not suitable.

Total Body Potassium

Total body potassium can be used to estimate the cellular contribution to body composition, by measuring gamma radiation emission from ^{40}K , a naturally occurring potassium isotope, present at constant concentrations of approximately 0.0118% of total potassium. Measurement of this isotope can be used to calculate total body potassium, and thus FFM and FM. Two assumptions are required: firstly that the potassium content of FFM is constant; secondly, that potassium is located almost exclusively in the FFM. (Forbes, Gallup, *et al.* 1961; Burmeister, 1965). This approach requires

expensive specialist equipment, but with access to equipment, it is relatively simple to conduct and safe. It has largely been superseded by newer techniques.

Imaging methods

Imaging techniques have the advantage of not only measuring body fat content in the whole organism, but also provide detailed information on fat distribution. The main approaches make use of dual energy x-ray absorptiometry (DEXA), computed tomography (CT) and magnetic resonance imaging (MRI) modalities.

DEXA makes use of the differential attenuation of X-ray radiation by bone, adipose and non-adipose soft tissue. The advantages are that it is quick to carry out and is relatively operator independent. It also provides information on total adipose tissue including both specific adipose depots and also any other fatty tissue in ectopic sites. Measurement of bone mass allows a more accurate assessment of body composition using the 4-compartment model. However, it can be expensive and requires exposure to ionising radiation. X-ray exposure is low, but this still limits its usefulness in certain populations such as children or pregnant women.

Whole-body CT imaging provides similar information; the relative attenuation of X-rays in different tissue types allows generation of images in grey-scale. The relative 'greyness' of each pixel in the image corresponds to the number of Hounsfield units (HUs) of that tissue. Adipose tissue appears white, with HUs between -190 to -30; muscle is denser, appears darker and has HUs in the positive range. It is therefore possible to use image analysis

software to set an arbitrary cut-off to identify which areas of the image have low attenuation and vice versa. The advantage of whole-body CT is that smaller discrete adipose depots can be quantified, such as intra-muscular lipid infiltration, in addition to measurement of specific adipose depots; data analysis is relatively operator independent, although it does require regions of interest to be identified and thresholds to identify adipose to be set. However, it can be very costly, with significant data analysis requirements and also requires significant exposure to x-ray radiation.

Magnetic Resonance Imaging allows similar assessments of adipose tissue volume and organ lipid content but avoids radiation exposure. Similar image analysis techniques can be applied to MR images as those used for analysis of CT-derived data. MR allows increased resolution of soft-tissue and thus more accurate delineation of adipose tissue depots.

1.1.3. Assessment of body composition in pregnancy

Changes in maternal body weight and composition have been associated with adverse pregnancy outcomes, particularly in obese women (Kiel, Dodson, *et al.*, 2007). It is therefore important to have a robust means of determining body composition during pregnancy. However, many commonly used means of assessment of body composition rely on determination of total body water, and are thus influenced by the relative hydration status of the subject. During pregnancy there is expansion of maternal body water due to increased blood volume, tissue growth and amniotic fluid, estimated to contribute 6-10L (Hytten., 1985). Consequently, methods which rely on accurate assessment of total body water are not valid. Although equations have been generated to account for a gestational change in composition and density of fat-free mass (van Raaj, Peek, *et al.* 1988); these were derived from a small number of normal weight women based on underwater weights at increasing gestation. Their applicability to a heterogeneous population is not clear and may be affected by differing body compositions at different gestations (Jaque-Fortunato, Khodiguian, *et al.* 1996).

An additional issue is that change in body composition requires an accurate assessment of a baseline measure. There are few data on early changes in body composition; it is likely to be slightly altered by early gestation, with small contributions from fetus and placenta, as well as expanded total body water, although some studies have suggested that there is in fact little change in body composition over the first trimester (Fattah, Farah, *et al.* 2010). Post-partum measurements are also unlikely to reflect a true pre-pregnancy state.

One approach has been to use a more complex model for body composition, with up to 4 compartments (fat mass, and fat-free mass composed of bone mass, body water and protein or muscle mass). Use of the 4-compartment model requires assessment of the bone mass of subjects; this can be done by DEXA and is therefore unsuitable unless subjects are assessed pre-pregnancy, as DEXA is unsafe to use in pregnancy. There is good correlation between 3- and 4-compartment models (Hopkinson, Butte, *et al.* 1997; Koop-Hoolihan, Van Loan, *et al.* 1999). Three compartment models have the advantage of not requiring assessment of pre-pregnancy bone mass. However, all approaches require certain assumptions to be made and are subject to a significant degree of variability within populations.

1.1.4. Quantifying intra-organ lipid content

In addition to quantification of adipose tissue depots, intra-organ lipid content is of increasing interest due to the observed association between intra-hepatic and intra-myocellular lipid content and insulin resistance, particularly in the context of obesity (Seppälä-Lindroos, Vehkavaara, *et al.* 2002). Biopsy is the current gold standard for direct quantification of intra-hepatic and intra-muscular lipid content. However, this is an invasive technique which places a high burden on research participants and is less suitable for longitudinal studies.

Imaging allows accurate, non-invasive quantification of organ lipid content, in particular ultrasound, CT and MR techniques including ¹H-MR spectroscopy. Ultrasound is commonly used to assess lipid infiltration in organs such as liver and to grade hepatic steatosis. Data can be very operator dependent but the technique is relatively cheap and accessible, which makes it a clinically useful modality for the assessment of patients with conditions such as hepatic steatosis (Ma, Holalkere, *et al.* 2009). CT images can be used to determine the relative lipid content of organs as well as identifying adipose depots. Lower mean attenuation of an organ correlates with lipid content, however, a high glycogen content can also affect attenuation and so relative rather than absolute lipid content is often generated (eg the ratio of attenuation of liver to that of spleen). Both ultrasound and CT provide qualitative rather than quantitative information about organ lipid content.

¹H-MR spectroscopy makes use of differing resonance frequencies of protons which are components of different molecules, usually metabolites such as

amino acids or creatine, and generates a plot of proton resonance relative to a known reference standard e.g. water. Peaks which correspond to specific molecules are identifiable at specific resonance frequencies, corresponding to the degree of shift from the standard. Data is generated from a specified voxel or region of interest within the organ, with signal intensity relative to the quantity of metabolite present in that voxel. Thus quantitative data regarding organ lipid content is obtained by the use of magnetic resonance spectroscopy (MRS). MRS has been thoroughly validated: MRS data correlates closely with lipid content of phantoms containing known lipid concentrations (Goodpaster, Stenger, *et al.* 2004) as well as with biopsy data from specific organs (Szczepaniak, Babcock, *et al.* 1999; Sharma, Kitajima, *et al.* 2011; Springer, Machann, *et al.* 2010). The main drawback of MRS is that it is costly to carry out, limiting its use to research studies rather than routine clinical practice.

Certain practical limitations can also influence the use of MRS. Firstly, MRS requires a uniform magnetic field, and uneven loading of the subject within the field can affect data capture. The process of shimming can compensate for this to a certain extent, however, patient positioning can also have a significant effect on magnetic field distortion. Therefore if a specific patient position is required, the acquisition of spectroscopy data can be affected. Secondly, data must be acquired from as homogeneous a voxel as possible. In liver, this requires avoidance of vessels, but also avoidance of lateral placement to avoid inclusion of subcutaneous or lung tissue in the voxel, particularly taking breathing motion into consideration.

1.1.5. Assessing Insulin Sensitivity/Resistance

A major consequence of excess adiposity is dysregulation of metabolism culminating in systemic resistance to the action of insulin. Insulin resistance (IR) contributes to the pathophysiology of several clinical conditions including metabolic syndrome & type II diabetes mellitus (Reaven, 1988), and can be defined as a reduction in the effectiveness of insulin at lowering extracellular glucose. Insulin resistance has been defined clinically as ‘a syndrome that is associated with a clustering of metabolic disorders, including non-insulin-dependent diabetes mellitus, obesity, hypertension, lipid abnormalities, and atherosclerotic cardiovascular disease’ (De Fronzo & Ferrannini, 1991).

Assessment of insulin sensitivity/resistance is essential as it is central to the diagnosis of specific conditions but also because identification of insulin resistant subjects at higher risk of development of frank disease facilitates early intervention and therapy. A large number of definitions of metabolic syndrome exist from a number of national and international institutions such as WHO, IDF, EGIR, ADA, SIGN, and NICE, each making use of differing measures of insulin resistance. However, common to all definitions is the concept that insulin resistance underpins the pathogenesis of type II diabetes mellitus, and is associated with an adverse metabolic and cardiovascular phenotype, and clinical features such as hyperlipidaemia, hypertension and obesity (Alberti, 2005).

Multiple methods of quantification of insulin resistance exist; the gold standard is the Hyperinsulinaemic Euglycaemic Clamp (HEC). The choice of

assessment depends on the study context, time available and cost. While clamp techniques provide an accurate assessment of IR, they are moderately invasive and time-consuming; therefore other methods have been developed which are simpler to conduct and have increased applicability at a population level.

Firstly, single measurements can be employed, at their most basic making use of measures of fasting glucose and insulin. These can be diagnostic in their own right, for example, fasting glucose > 7.0mmol/L is diagnostic of diabetes mellitus (World Health Organization criteria). An elevated fasting insulin can be indicative of insulin resistance (Laasko, 1992) although caution has to be taken in certain clinical circumstances, eg type 2 diabetes mellitus, where beta cell failure and reduced insulin secretion is a feature. Improved correlation with clamp assessments of IR can be derived from a combination of fasting glucose and insulin measurements, using models such as the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) (Matthews, Hosker, *et al.* 1985) and QUantitative Insulin sensitivity Check Index (QUICKI) (Katz, Nambi, *et al.* 2000). These methods make the assumption that hepatic and peripheral IR are equivalent.

HOMA-IR is calculated as follows:

$$(Fasting\ glucose\ (mmol/L) \times Fasting\ insulin\ (\mu U/mL))/22.5$$

QUICKI is calculated as follows:

$$1 / (\log(fasting\ insulin\ \mu U/mL) + \log(fasting\ glucose\ mg/dL))$$

Both of these indices have been evaluated against HEC and show reasonable correlation with clamp-derived IR. Their major advantage is that they require only a single fasting sample from the patient, and are thus more suitable for use in the general population.

A further index which can be used is that of Matsuda (Matsuda & deFronzo, 1999), which uses measurements obtained during an oral glucose tolerance test, ie fasting and 2 hour samples. This has the advantage of assessing both hepatic IR (fasting state) and peripheral IR (fed state post-glucose challenge) and is calculated thus:

$$10,000/\sqrt{([fasting\ glucose(mg/dL) \times fasting\ insulin(\mu U/mL)] \times [mean\ glucose(mg/dL) \times mean\ insulin\ during\ OGTT(\mu U/mL)])}$$

This index correlates closely with that of HOMA-IR ($r = 0.92$) and with SI_{clamp} ($r = 0.74$) (Matsuda & deFronzo, 1999), and provides a more detailed assessment of whole body insulin sensitivity from easily obtainable samples during an OGTT rather than a snapshot of the fasting state alone.

More detailed assessments of IR can be derived by the use of minimal modelling techniques (Saad, Anderson, *et al.*, 1994). These include the Frequently Sampled IV Glucose Tolerance Test (FSIVGTT) and the Continuous Infusion of Glucose with Model Assessment (CIGMA). These techniques make use of multiple samples to more accurately model glucose disposal in both hepatic and peripheral compartments using mathematical algorithms. These are useful in the context of a research study but are less practical in a general clinical context.

The use of both and glucose and insulin challenge provides further assessment of metabolic regulation; techniques includes the insulin tolerance test, involving measuring the decline in circulating glucose concentrations following an insulin bolus, and the insulin sensitivity test (Greenfield, Doberne, *et al.* 1981) which uses octreotide, a potent somatostatin analogue, to suppress endogenous insulin followed by a fixed infusion of insulin with a defined glucose load. The advantage of this technique is that it negates the beta-cell contribution to peripheral insulin sensitivity.

As previously stated, the hyperinsulinaemic euglycaemic clamp remains the gold standard method for assessing insulin sensitivity. A constant insulin infusion provides supra-physiological plasma insulin concentrations; this has the effect of stimulating glucose uptake peripherally, suppressing endogenous glucose production by the liver as well as affecting other insulin-sensitive pathways. A concomitant glucose infusion is titrated to maintain plasma glucose concentrations at euglycaemia (a common target is 4.7mmol/L). The rate of glucose infusion in steady state conditions is a direct measure of whole body glucose disposal and represents basal glucose uptake as well as uptake by all insulin-sensitive tissues. The greater the rate of glucose infusion, the greater the subject's sensitivity to insulin. This is expressed as M , in mg glucose/kg/min. M can also be expressed as mg/kg fat free mass/min (mg/kg_{FFM}/min). Fat free mass is largely composed of skeletal muscle, which is the biggest contributor to insulin-sensitive glucose uptake; therefore expressing whole body glucose uptake in this way is more specific for insulin sensitive tissues. This has to be interpreted with caution in pregnancy as estimates of fat mass and fat free mass are less accurate in pregnancy as discussed in section 1.1.3; while expressing M per kg fat free

mass expresses substrate utilisation by insulin sensitive tissues, this could be influenced by less accurate assessment of total body fat, particularly in the third trimester due to the influence of the fetoplacental unit. However, expression of M per kg total body mass could lead to a potential underestimation of insulin sensitive glucose disposal, particularly in lean women, as they have proportionately greater lean body mass per kg compared to obese subjects.

An index of insulin sensitivity can also be derived from the M value, by expressing glucose disposal as a function of insulin concentration, M/I . This expresses insulin sensitivity relative to the quantity of glucose disposal achieved per unit insulin.

Clamp studies also provide the means to study additional metabolic pathways such as endogenous glucose production, lipolysis and also energy expenditure by the concomitant use of techniques such as isotopic tracers or indirect calorimetry. Tracer studies utilise either stable or radiolabelled isotopes. Stable isotopes include 6,6-d₂ glucose and 1,1,2,3,3-d₅ glycerol which contain deuterium labels and are thus identifiable using mass spectrometry techniques. Lack of radioactivity makes them particularly suitable for use in most study populations, including pregnant women. In the work described in this thesis, we have employed glucose and glycerol stable isotope tracers in the context of a hyperinsulinaemic euglycaemic clamp to quantify insulin resistance, endogenous glucose production and rate of lipolysis in obese pregnant women.

1.2. Adipose tissue biology in health

Adipose tissue is an organ of mesodermal origin whose primary function was until recently considered to be purely one of energy storage as fat in the form of triglyceride. However, it has become apparent in recent years that adipose tissue is not only a highly active endocrine organ, but has specific functions depending on its anatomical location.

1.2.1. Anatomy

There are two types of adipose, brown adipose tissue (BAT) and white adipose tissue (WAT). WAT is the major type of adipose in human adults, and is the depot which is expanded in obesity. In humans, BAT is predominantly found in the fetus and neonate and is principally involved in thermogenesis. BAT shares a common embryological origin with myocytes, is multilocular and dense in mitochondria (Seale, Bjork, *et al.* 2008). BAT comprises less than 1% of total adipose tissue in adult humans, but it may have a role in maintaining energy homeostasis. However, it has recently been shown that brown adipose tissue is present in adults (Cypess, Lehman, *et al.* 2009), can be activated in adults in response to cold (Virtanen, Lidell, *et al.* 2009) and that the amount of BAT present is inversely proportional to body mass index which may reflect a reduction in the requirement for non-shivering thermogenesis in obese subjects or a predisposition to adipose accumulation via a pre-existing defect in the ability to dispose of excess calories (Cypess, Lehman, *et al.* 2009; Vijgen, Bouvy, *et al.* 2010). It has also been shown that there is a population of beige or 'brite' adipocytes which arise from white adipose but express low levels of UCP-1, a classical marker of brown adipocytes. These cells respond to stimulation in a similar manner

to brown adipocytes and may be the origin of cold-responsive brown adipose in humans (Wu, Boström, *et al.* 2012).

Composed of adipocytes, supporting stromal cells and a vascular network, in humans, the major WAT depots are subcutaneous and visceral, although there are also some smaller adipose deposits in bone marrow and intramuscular. Subcutaneous adipose tissue displays regional characteristics, depending on anatomical location, e.g. abdominal, gluteal, or thoracic. Adipocytes in WAT derived from mesenchymal stem cells begin to form around week 17 of fetal development, though the majority of fetal adipose tissue deposition occurs in the third trimester (Symonds, Mostyn, *et al.* 2003). While fetal adipose displays characteristics of both WAT and BAT, in adult life, the majority is WAT. Adipose tissue also contains lymph nodes with a locally adaptive immune cell content and stromal cells: macrophages, fibroblasts and endothelial cells. These cells are thought to have a local regulatory role in adipose tissue function, and along with adipocytes themselves, are a source of the many secretory products of adipose tissue.

1.2.2. Biochemical properties of adipose tissue

1.2.2.1. Lipid storage & release

One of the major functions of the adipocyte is to store energy as intracellular triglyceride. Circulating protein-bound lipid in the form of chylomicrons or very low-density lipoprotein (VLDL) is hydrolysed by lipoprotein lipase (LPL), bound to the endothelial surface of adipose tissue capillaries via heparin sulphate proteoglycans. Following hydrolysis, non-esterified fatty acid is taken up by the adipocyte by a number of binding proteins, e.g. fatty acid transport protein (FATP), converted to fatty acyl CoA and subsequently re-esterified with glycerol-3-phosphate in the endoplasmic reticulum to form triacylglyceride (TAG). The rate limiting step in TAG synthesis is catalysed by diglycerol acyl transferase (DGAT), an endoplasmic reticulum membrane bound protein. TAG is stored usually in a single large anhydrous lipid droplet within the cytoplasm of the adipocyte.

Lipid storage is under endocrine regulation which controls the relative rates of lipid synthesis and lipolytic mechanisms through regulation of lipid turnover at the intracytoplasmic lipid droplet.

Access to the lipid droplet is regulated by perilipins, which prevent access to triglyceride by hormone-sensitive lipase (HSL), initially considered the rate-limiting enzyme in the lipolytic cascade. Under basal conditions, HSL is located diffusely throughout the cytoplasm, however, upon phosphorylation, which occurs physiologically in response to fasting, it is translocated to the surface of the lipid droplet (Egan, Greenberg, *et al.* 1992; Brasaemle, Levin, *et al.* 2000)), where interaction with phosphorylated perilipin allows access to the lipid droplet (Sztalryd, Xu, *et al.* 2003). HSL has specificity for both TAG and DAG; glycerol and the last remaining fatty acid are released by the action of monoacylglycerol lipase. A third lipase, adipose triglyceride lipase (ATGL), has also been identified and has a role in the initiation of lipolysis. Both ATGL and HSL are required for normal full induction of lipolysis (Zimmerman, Strauss, *et al.* 2004). Following sequential hydrolysis of a molecule of triglyceride, one molecule of glycerol and three of fatty acid are released into the circulation. Fatty acid binding protein (FABP) is important in intracellular transport and release of FFA from the lipid droplet to the plasma membrane (Baar, Dingfelder, *et al.* 2004); FFA are subsequently exported to the liver bound to albumin, where they undergo beta-oxidation; glycerol is exported from the adipocyte via aquaporin 7 (Maeda, Funahashi, *et al.* 2004), and can be used as a substrate for gluconeogenesis in the liver following uptake and phosphorylation by glycerol kinase.

The regulation of lipid turnover is under significant hormonal control and which pathway dominates depends on whether the individual is in an energy requiring or energy-replete state.

The principle hormone promoting lipid storage is insulin. Insulin acts via a membrane-bound tyrosine kinase receptor. Phosphorylation of the intracellular domains upon insulin binding activates the phosphoinositide-3-kinase (PI3K) signalling cascade, resulting in the translocation of the Glucose Transporter (GLUT)4 to the cell surface, enhanced lipid esterification and inhibition of lipolytic enzymes. Insulin-dependent glucose uptake is increased following translocation of GLUT4 (Slot, Geuze, *et al.* 1991); upon entry to the adipocyte, glucose is phosphorylated by glucokinase and enters the lipid synthesis pathway. Enhanced production of glycerol contributes to lipid accumulation as it esterifies with the accumulating free fatty acid. Phosphorylation of insulin-receptor substrate-1 (IRS-1) promotes association between the plasma membrane and PI3K, leading to activation of PI3K and subsequently the activation of protein kinase B/Akt. PKB activates phosphodiesterase 3b which inhibits HSL by reducing cAMP levels.

The principal hormones promoting lipolysis are the catecholamines, of which the principle adrenoceptors of adipocytes are beta 1 and 2; beta-3 adrenoceptors are also capable of promoting lipolysis but are thought to be of less importance in humans. Adrenaline and noradrenaline signal via cAMP, and activate PKA. This kinase then rapidly phosphorylates and activates HSL. Polymorphisms in the adipocyte beta-2 adrenoceptor have been linked to obesity (Large, Hellström, *et al.* 1997).

A number of other hormones have regulatory activity in the adipocyte. Glucagon stimulates lipolysis *in vitro* (Langslow & Hales, 1969); however, *in vivo* studies using microdialysis in humans have not shown an effect on lipolysis (Bertin, Arner, *et al.* 2001; Gravholt, Møller, *et al.* 2001). Atrial

natriuretic peptide (ANP) stimulates lipolysis in a cGMP dependent mechanism via cGMP-dependent protein kinase (PKG) in response to exercise (Moro, Pillard, *et al.* 2008). Thyroid hormones can also act to enhance the effect of catecholamines on lipolysis by increasing receptor availability and reducing secondary messenger turnover via reduced phosphodiesterase activity (Hellström, Wahrenberg, *et al.* 1997).

Glucocorticoids also have a role in maintaining lipid storage: *in vitro*, treatment of isolated adipocytes with cortisol reduces basal and isoprenaline-stimulated lipolysis (Ottosson, Lönnroth, *et al.* 2000); dexamethasone has a stimulatory effect on LPL mRNA synthesis and activity (Fried, Russell, *et al.* 1993). There are regional variations in response to glucocorticoid; omental adipose has a greater density of glucocorticoid receptors than subcutaneous adipose (Pedersen, Jønler, *et al.* 1994). Recent evidence has highlighted the importance of local regulation of availability of glucocorticoids through the activity of 11-beta-hydroxysteroid dehydrogenase type 1 (11 β -HSD1). This enzyme converts inactive cortisone to active cortisol (and also the reverse reaction) and thus enhances local tissue availability of active glucocorticoid (Hughes, Webster, *et al.* 2008).

1.2.2.2. Endocrine functions of adipose tissue

In the last 20 years there has been a paradigm shift from adipose tissue being viewed as an inert lipid storage organ to that of an active endocrine organ. Many hormones, with endocrine and paracrine actions are produced by adipose tissue, both by adipocytes themselves and also by the supporting stromal cells. Secretory products of adipose tissue are collectively known as adipokines, and include a diverse range of proteins such as the classical adipokines, leptin and adiponectin, as well as inflammatory cytokines such as interleukin 6 and tumour necrosis factor alpha.

Leptin is a 16kDa hormone that has pleiotropic effects on multiple organs. It is primarily synthesized by white adipose tissue (Zhang, Proenca, *et al.* 1994) but is also produced by ovary (Cioffi, Van Blerkom *et al.* 1997), pituitary (Jin, Burguera, *et al.* 1999), placenta (Señarís, Garcia-Caballero, *et al.* 1997) and also in baboon fetal lung (Henson, Swan, *et al.* 2004). It has a role in the regulation of appetite, satiety, reproductive function, body mass and immune regulation (Friedman & Halaas, 1998; Chan, Matarese, *et al.* 2006; Kelesidis, Kelesidis, *et al.* 2010). Circulating levels of leptin are proportional to fat mass (Maffei, Halaas, *et al.* 1995; Considine, Sinha, *et al.* 1996; Rönnemaa, Karonen, *et al.* 1997) and correlate inversely with insulin sensitivity independently of fat mass (Silha, Krsek, *et al.* 2003).

Adiponectin is a 30kDa protein which circulates in a multimeric form in plasma and is secreted by adipocytes (Scherer, Williams, *et al.* 1995) but also by salivary gland epithelial cells (Katsiougianis, Kapsogeorgou, *et al.* 2006), leucocytes (Crawford, Peake, *et al.* 2010) and skeletal muscle (Liu, Chewchuk,

et al. 2009). It has an inverse pattern of expression to leptin: it is synthesized in adipose tissue but is inversely correlated with fat mass, particularly in men (Zhang, Holt, *et al.* 2005); adiponectin levels correlate directly with insulin sensitivity (Hotta, Funahashi, *et al.* 2001) and treatment with adiponectin can reverse the insulin resistance of obesity (Yamauchi, Kamon, *et al.* 2001).

There are a number of other endocrine products of adipose tissue. These are summarised in Table 1. Among these, inflammatory products are of importance in the context of obesity as their synthesis and secretion become dysregulated. However, their role in the physiological regulation of adipose tissue function is not clear.

Adipokine	Functional effects	Reference
Pro-inflammatory Cytokines		
Tumor necrosis factor (TNF) α	Stimulates lipolysis	Zhang, <i>et al.</i> 2002
	Increases insulin resistance via interference with IRS1 downstream signalling	Hotamisligil, <i>et al.</i> 1996
Monocyte chemoattractant protein (MCP)-1	Impairs pre-adipocyte differentiation	Isakson, <i>et al.</i> 2009
Interleukin (IL)-6	Promotes monocyte recruitment into WAT	Kanda, <i>et al.</i> 2006
IL-8	Stimulates lipolysis	Trujillo <i>et al.</i> 2004.
IL-1 β	Potent chemoattractant	Bruun, <i>et al.</i> 2001
	Impairs insulin signalling by inhibition of phosphorylation of IRS1	Lagathu, <i>et al.</i> 2006
	Cytotoxic effects on pancreatic islet	Bergmann, <i>et al.</i> 1992
Anti-inflammatory Cytokines		
IL-10	Broad anti-inflammatory activity	Couper, <i>et al.</i> 2008
	Altered ATM phenotype	Fujisaka, <i>et al.</i> 2009
	May protect against development of IR	Lumeng, <i>et al.</i> 2007
IL-1Ra	Antagonises IL1	Arend, <i>et al.</i> 2001
Hormones		
Leptin	Central regulation of appetite	Kalra, <i>et al.</i> 1999
	Stimulates fatty acid oxidation in liver and skeletal muscle	Minokoshi, <i>et al.</i> 2001
	Inhibits insulin secretion from pancreas	Emilsson, <i>et al.</i> 1997
	Induces secretion of IL1-Ra	Dreyer, <i>et al.</i> 2002
Adiponectin	Stimulates fatty acid oxidation and glucose utilization	Yamauchi, <i>et al.</i> 2002
	Promotes adipocyte differentiation	Fu, <i>et al.</i> 2005
	Reverses obesity-associated IR	Yamauchi, <i>et al.</i> 2001
	Improves hepatic IS	Stefan, <i>et al.</i> 2003
	Induces secretion of IL-10 & IL1Ra	Wolf, <i>et al.</i> 2004
Visfatin	Regulates β -cell function in pancreas	Revollo, <i>et al.</i> 2007
	Upregulates MCP-1 in adipocytes	Sommer, <i>et al.</i> 2010
Resistin	Impairs glucose tolerance in mice	Steppan, <i>et al.</i> 2001
	Pro-inflammatory cytokine in humans	Nagaev, <i>et al.</i> 2006
Apelin	Regulates insulin sensitivity and adiposity in rodents	Higuchi, <i>et al.</i> 2007
Omentin	Insulin sensitising	Yang, <i>et al.</i> 2006
Other factors		
Retinol binding protein (RBP)-4	Increases adipose tissue or whole body? insulin resistance in animal studies	Yang <i>et al.</i> 2005
Plasminogen activator Inhibitor (PAI) -1	Involved in regulation of adipose tissue development	Morange, <i>et al.</i> 2000
Angiotensinogen	Increases fat mass and hypertension in transgenic overexpressing mice	Massiéra, <i>et al.</i> 2001
Prostaglandins	Inhibit adipocyte lipolysis	Fredholm, <i>et al.</i> 1973

Table 1.1 Summary of major adipokines & their actions. (Adapted from Denison, Roberts, Barr & Norman, Reproduction. 2010)

1.2.3. Depot specific characteristics of adipose tissue

There are regional differences in the characteristics of adipose depots, which may explain why certain adipose tissue distributions have differing effects on cardiovascular and metabolic risk. Visceral adipose tissue refers to intra-abdominal adipose, including omentum, mesenteric deposits as well as retro-peritoneal deposits such as peri-renal adipose. Depot-specific characteristics are both anatomical and functional; visceral adipocytes tend to be smaller than subcutaneous adipocytes, and there is greater stromal cell infiltration into visceral adipose depots, particularly in obesity (Harman-Boehm, Blüher, *et al.* 2007). Adipocyte cell size is important with respect to metabolic risk, though it seems this is most important in the omental depot (O'Connell, Lynch, *et al.* 2010).

Omental adipose tissue is considered more metabolically active compared with subcutaneous adipose; there is evidence to suggest it has a greater secretory capacity compared with subcutaneous adipose (Hocking, Wu, *et al.* 2010), as well as an enhanced lipolytic response to catecholamines, particularly in obesity (Hoffstedt, Arner, *et al.* 1997) and a differential response to regulation by glucocorticoids (Lee, Gong, *et al.* 2011). It has been proposed that its anatomical location, with venous drainage into the portal circulation allows it to act as a metabolic sensor with direct hepatic access to free fatty acids, which drives hepatic insulin resistance in obesity (Björntorp, 1990; Kabir, Catalano, *et al.* 2004).

In contrast, subcutaneous adipose, with larger adipocytes, may have more of a role in lipid storage, comprising approximately 80% of total body adipose.

Subcutaneous adipose explants secrete increased angiogenic factors compared with visceral adipose, which may facilitate its expansion as a storage depot (Hocking, Wu, *et al.* 2010; Gealekman, Guseva, *et al.* 2011). Subcutaneous adipose is thought to be the main contributor to circulating leptin concentrations; leptin mRNA is greater in subcutaneous compared with omental adipose tissue, with a marked gender specific effect: in men, leptin mRNA in subcutaneous adipose tissue is approximately twice that of omental adipose, but in women, there is a 5-fold increase in leptin mRNA in subcutaneous compared with omental adipose (Montague, Prins, *et al.* 1998; Van Harmelen, Reynsdottir, *et al.* 1998).

Regional characteristics of adipose tissue depots show gender-specific patterns as well: differences in adipose tissue distribution between men and women have long been recognised (Krotiewski, Björntorp, *et al.* 1983) and may account for the differences in cardiovascular risk between males and females. Men tend toward deposition of adipose in the central or upper body, 'android' distribution, whereas women tend to distribute fat on hips, thighs and buttocks, a 'gynoid' distribution. This distinction is important in determining metabolic risk: there is an association between android but not gynoid obesity and risk of metabolic syndrome, diabetes and cardiovascular disease (Kissebah, Vydelingum, *et al.* 1982; Canoy, Boekholdt, *et al.* 2007). Molecular differences in regional adipose characteristics and response to sex steroids may account for these different risks: for example, *in vitro*, dehydroepiandrosterone (DHEA) preferentially stimulates lipolysis in omental adipose in men, but subcutaneous adipose in women (Hernández-Morane, Pérez-de-Heredia, *et al.* 2007); progesterone treatment in rats altered body fat distribution and adipokines expression in female but not male

animals (Stelmanska, Kmiec, *et al.* 2012). In ovariectomised mice, treatment with androgens leads to visceral fat accumulation (McInnes, Corbould, *et al.* 2006). In post-menopausal women, adipose distribution become more central compared with pre-menopausal women, but this can be counteracted to a certain extent by the use of combined HRT (Gambacciani, Ciaponi *et al.* 1997).

1.3. Alterations to adipose tissue function in obesity

Obesity in its simplest definition is the presence of excess adipose tissue. However, it has become clear that over-expansion of the adipose depots leads to abnormal adipose function and systemic consequences to metabolic regulation. Clinically this manifests as systemic insulin resistance, impaired regulation of glucose homeostasis and vascular dysfunction, contributing to the development of metabolic syndrome, diabetes, hypertension and increased cardiovascular disease risk. However, the molecular mechanisms which contribute to this increase in risk are not yet fully understood but involve a complex interaction between the secretory products of adipose tissue, local effects on adipose tissue and systemic effects on other organs, particularly liver, skeletal muscle and brain.

1.3.1. Adiposity and Origins of Insulin resistance

Increased adipose tissue has been associated with the development of resistance to the action of insulin for many years and many epidemiological studies have shown a strong positive correlation between markers of adiposity including BMI (Abbasi, Brown *et al.* 2002; Farin, Abbasi, *et al.* 2006; Schindler, Cardenas, *et al.* 2006) or increased WHR (Toft, Bønna, *et al.* 1998) and measure of IR. It is likely that there is a self-propagating mechanism by which excess adiposity leads to local intra-adipose insulin resistance, originating with the lipotoxic effects of nutrient excess, with accompanying oxidative stress triggering adipose inflammation and exacerbating adipose dysfunction.

During fat mass expansion, at the level of the adipocyte, there is initial expansion of the lipid droplet, with concomitant adipocyte hypertrophy (Hirsch & Batchelor, 1976). This is accompanied by changes in gene expression, with down regulation of genes associated with lipogenesis and fatty acid turnover (Roberts, Hodson, *et al.* 2009; McQuaid, Hodson, *et al.* 2011), as well as upregulation of inflammatory cytokines such as TNF α (Hotamisligil, Shargill, *et al.* 1993). These early changes in adipocyte characteristics interact with the supporting stromal cells present in adipose tissue to lead to further self-propagating adipose tissue dysfunction. Consequent spillover of factors such as pro-inflammatory cytokines and excess fatty acids occurs not simply due to excess fat mass but to altered behaviour of the component cells. Ultimately this leads to systemic diversion of lipid to ectopic sites which interferes with insulin function in these organs, thus propagating systemic insulin resistance.

1.3.1.1. Origins of Insulin Resistance: Inflammation

Obesity has been described as a low-grade, chronic inflammatory condition. There is evidence for elevated circulating pro-inflammatory cytokines, including TNF α (Kern, Saghizadeh, *et al.* 1995), IL-6 (Roytblat, Rachinsky, *et al.* 2000), MCP-1 (Kim, Park, *et al.* 2006) and elevated acute phase proteins such as CRP (Visser, Bouter, *et al.* 1999). In 1993, work by Hotamisligil and colleagues showed that not only was adipose a source of TNF α , but that this elevated TNF α had a role in the molecular pathogenesis of obesity-associated insulin resistance (Hotamisligil, Shargill, *et al.* 1993). Subsequently, it was shown that not only was adipose tissue responsible for production of a myriad of secretory proteins, but that there was a direct correlation between inflammatory status and insulin resistance (Kern, Ranganathan, *et al.* 2001). It is likely that initiation of the inflammatory response in obesity lies in adipose tissue possibly as a response to stimulation by endoplasmic reticulum stress accompanying adipocyte overexpansion and lipid accumulation (Özcan, Cao, *et al.* 2004) but that this process becomes self-propagating and triggers chronic systemic inflammation.

In 2003, Weisberg and colleagues demonstrated a correlation between obesity and infiltration of adipose tissue by macrophages/increased macrophage density and suggested that it was the increased macrophage population that was responsible for the elevated pro-inflammatory cytokines (Weisberg, McCann, *et al.* 2003; Xu, Barnes, *et al.* 2003). However, adipocytes themselves are also capable of secretion of TNF α . In adipose tissue from lean subjects, macrophages are present, though sparse. These are thought to be resident, bone-marrow derived macrophages. Macrophage density is

significantly increased in adipose tissue from obese subjects. These are a specific subset of resident macrophages rather than deriving from circulating monocytes (Weisberg, McCann, *et al.* 2003), and have a specific phenotype, neither M1- or M2- specific. M1-like macrophages are classically activated, pro-inflammatory cells, whereas M2-like macrophages are alternately activated, pro-resolution cells. High-fat feeding in a rodent model induced a change in phenotypic characteristics of adipose tissue macrophages from M2 to M1 (Lumeng, Bodzin, *et al.* 2007). In humans, isolated macrophages from adipose tissue of obese subjects have an M2-like phenotype but have the capacity to produce pro-inflammatory cytokines, a more M1-like property (Zeyda, Farmer, *et al.* 2007).

Mechanisms by which inflammation leads to insulin resistance are varied and are secondary to local effects in adipose tissue, liver and skeletal muscle. In adipose tissue, TNF α treatment of adipocytes leads to inhibition of insulin receptor signalling (Hotamisligil, Murray, *et al.* 1994; Liu, Spelleken, *et al.* 1998). Treatment with IL6 led to long term downregulation of expression of GLUT-4, IRS-1 and PPAR γ (Rotter, Nagaev, *et al.* 2003). In liver, pro-inflammatory cytokines activate NF- κ B signalling and induce insulin resistance (Cai, Yuan, *et al.* 2006); treatment with IL-6 reduces hepatic IRS1 signalling (Klover, Zimmers, *et al.* 2003). Hepatic lipid infiltration in obesity also stimulates a local inflammatory response via oxidative stress (Peng, Rideout, *et al.* 2011). Similarly, IL-6 also reduces insulin signalling via IRS1 in skeletal muscle (Kim, Higashimori, *et al.* 2004). Interaction with other adipokines may also modulate the inflammatory response: visfatin is increased in obesity as well as other pro-inflammatory states such as arthritis (Berndt, Klöting, *et al.* 2005; Krzyzanowska, Mittermayer, *et al.* 2006;

Brentano, Schorr, *et al.* 2007) and may be involved in regulating insulin responsiveness via inflammatory mechanism including prostaglandin synthesis (Jacques, Holzenberger, *et al.* 2012).

1.3.1.2. Origins of Insulin Resistance: Adipokines

The endocrine products of adipose tissue also have a role in driving systemic metabolic dysregulation. The classic adipokines, leptin and adiponectin have multiple effects on normal metabolic homeostasis and appetite regulation as outlined in Section 1.2.2.2. In obesity, their expression and the systemic responses to them are altered significantly.

Typically, circulating leptin levels are increased in obese individuals (Maffei, Halaas, *et al.* 1995; Havel, Kasim-Karakas, *et al.* 1996; Considine, Sinha, *et al.* 1996). On a short-term basis, leptin enhances insulin sensitive pathways, including insulin-suppressed endogenous glucose production (Rosetti, Massilon, *et al.* 1997) although there may also be inhibition of some insulin-responsive pathways (Cohen, Novick, *et al.* 1996). Lipoatrophic individuals are insulin resistant, with elevated FFA and triglyceride; leptin levels are typically very low and treatment with leptin can improve insulin sensitivity in both mice (Shimomura, Hammer, *et al.* 1999) and humans (Petersen, Oral, *et al.* 2002). In contrast, in diet-induced obesity there is often severe insulin resistance despite high levels of leptin. However, this may reflect end-organ insensitivity to leptin. Altered permeability of the blood-brain-barrier to leptin may be a factor: hypothalamic signalling in response to leptin is reduced in response to diet-induced obesity in a murine model when given peripherally, although a response is retained although attenuated when it is

administered directly into the cerebrospinal fluid (CSF) (El-Haschimi, Pierroz, *et al.* 2000); in humans, there is a reduced CSF:serum leptin ratio in obese subjects (Caro, Kolaczynski, *et al.* 1999).

In contrast to leptin, adiponectin expression is decreased in overweight and obese subjects, including mice (Hu, Liang, *et al.* 1996) and human (Arita, Kihara, *et al.* 1999) but is also greatly reduced or absent in cases of lipoatrophy (Yamauchi, Kamon, *et al.* 2001). Treatment with adiponectin can reverse the insulin resistant phenotype of lipoatrophy (Yamauchi, Kamon, *et al.* 2001). Adiponectin exerts its effects on a number of tissues. In skeletal muscle, it upregulates expression of proteins of fatty acid transport and stimulates oxidative metabolism by activation of AMPK, p38 MAPK and PPAR α (Yoon, Lee, *et al.* 2006) as well as stimulating glucose uptake by increasing GLUT4 translocation (Ceddia, Somwar, *et al.* 2005) and enhancing mitochondrial biogenesis (Qiao, Kinney, *et al.* 2012). In liver, adiponectin is insulin-sensitising (Berg, Combs, *et al.* 2001); plasma adiponectin levels are negatively correlated with insulin-suppressed endogenous glucose production in humans (Stefan, Stumvoll, *et al.* 2003); adiponectin treatment leads to reduced hepatocyte gluconeogenesis in a murine model (Yamauchi, Kamon, *et al.* 2002). Reduced adiponectin in obesity is therefore likely to contribute to obesity-associated IR by absence of its insulin-sensitising actions. High-molecular weight adiponectin exists in a multimeric form of adiponectin, composed of 12-18 subunits; women tend to have greater high-molecular weight adiponectin than men (Pajvani, Du, *et al.* 2003). In particular, the high-molecular weight isoform of adiponectin is thought to be the most relevant to its insulin-sensitising actions (Pajvani, Hawkins, *et al.* 2004) and in women, the ratio of high-molecular weight:total adiponectin is

related to risk of diabetes independently of total adiponectin (Heidemann, Sun, *et al.* 2008).

Several other adipokines have been implicated in the pathogenesis of diet-induced insulin resistance, including omentin, apelin, resistin and RBP-4. Omentin is a novel adipokine which is highly expressed in visceral adipose and is thought to originate in the stromal vascular cells (Yang, Lee, *et al.* 2006). It acts to enhance insulin signalling and increases insulin-stimulated glucose transport in adipocytes (Yang, Lee, *et al.* 2006). Expression and plasma levels are decreased in obese insulin resistant subjects (de Souza Batista, Yang, *et al.* 2007) but this may reflect down regulation in response to hyperinsulinaemia in such subjects (Tan, Adya, *et al.* 2008).

In contrast, apelin is increased in obese subjects (Boucher, Masri, *et al.* 2005). In a murine model, treatment with apelin increased glucose uptake and improved glucose tolerance in obese mice (Dray, Knauf, *et al.* 2008) and increased expression in obesity may reflect a compensatory mechanism to counteract developing IR. Resistin is also increased in obesity (Steppan, Bailey, *et al.* 2001; Azuma, Katsukawa, *et al.* 2003). Administration of anti-resistin antibodies to mice led to an improvement in glucose tolerance and insulin response in a model of diet induced obesity (Steppan, Bailey, *et al.* 2001). In a murine resistin knock-out model, mice had reduced hepatic glucose production in response to fasting, but had better glucose tolerance following high-fat feeding (Banerjee, Rangwala, *et al.* 2004). Retinol binding protein 4 (RBP4) is also increased in insulin resistance (Yang, Graham, *et al.* 2005); overexpression of RBP4 leads to IR in a murine model (Yang, Graham, *et al.* 2005). RBP4 has been reported to be downregulated in subcutaneous

adipose in human obesity (Janke, Engeli, *et al.*, 2006) but upregulated in omental adipose and positively correlated with fasting glucose levels (Kelly, Kashyap, *et al.*, 2010) and insulin resistance (Graham, Yang, *et al.*, 2006). RBP4 may exert its effects on glucose homeostasis and insulin sensitivity by regulation of GLUT4 function (Chiefari, Paonessa, *et al.*, 2009).

1.3.1.3. Origins of Insulin Resistance: Effect of sex steroids

In non-pregnant women, there is an association between levels of sex steroids, oestrogens and progesterone, and insulin sensitivity. Insulin sensitivity varies across the menstrual cycle, particularly in the luteal phase and with a positive association between insulin resistance and levels of oestrogen and progesterone (Yeung, Zhang, *et al.*, 2010). Treatment with 17 β -estradiol improves skeletal muscle insulin sensitivity in ageing women (Moreno, Ordoñez, *et al.*, 2010); however, hormone replacement therapy (HRT) (oestrogen only and combined with progesterone) is associated with insulin resistance in post-menopausal women (Ryan, Nicklas, *et al.*, 2002). In women, androgen administration can induce insulin resistance, as can oestrogen treatment in males (Polderman, Gooren, *et al.*, 1994). Adipose tissue is also a source of extragonadal oestrogen although this appears to only leads to a significant rise in plasma oestrogen in obese males rather than obese pre-menopausal females (Zumoff, Strain, *et al.*, 1981).

In the context of polycystic ovarian syndrome (PCOS), there is an association with insulin resistance and hyperinsulinaemia (Dunaif, Segal, *et al.*, 1989). While there is also an association between PCOS and obesity which could contribute to this association, the effect appears to be independent of obesity,

although overweight and obese women with PCOS tend to be more insulin resistant than lean women with PCOS (Dunaif, Segal, *et al.* 1989). The cellular mechanisms implicated include alteration in insulin receptor function through altered serine phosphorylation, (Dunaif, Segal, *et al.* 1992) or decreased GLUT4 expression (Rosenbaum, Haber, *et al.* 1993). Whether this is due to an intrinsic defect in subjects with PCOS or an effect of the hormonal changes of this condition is not entirely clear. On one hand, androgen administration, particularly testosterone, appears to induce insulin resistance but treatment with anti-androgens does not always particularly improve insulin resistance. Similarly, the effect of PCOS on insulin sensitivity does not appear to correlate directly with androgen levels. It is likely that androgen may contribute to the insulin resistance of PCOS but other factors are also required.

That an interaction between oestrogen, progesterone, androgen, adiposity and insulin resistance exists seems established. However, the direct molecular mechanisms are not yet fully understood and while there appears to be a degree of independence between each of these variables, their relative contributions in differing clinical contexts remains elusive, particularly in separating the effect of obesity on sex steroid levels and vice versa.

1.3.2. Consequences of Adipose Insulin Resistance

In association with the development of insulin resistance in adipose tissue, there is spillover of both excess nutrients such as FFA as well as dysregulated secretion of adipokines and pro-inflammatory cytokines as outlined above. These factors have a number of effects on other organs to generate systemic insulin resistance and the pathophysiological features associated with obesity.

In liver, excessive supply of FFA in the face of reduced adipose storage capacity due to insulin resistance leads to accumulation of intracellular lipid, although this is not always correlated with the degree of obesity *per se* (Seppälä-Lindroos, Vehkavaara, *et al.* 2002). There is increased VLDL production, due to both increased supply of FFA as well as increased *de novo* lipogenesis (Lewis, Carpenter, *et al.* 2002), contributing to systemic hyperlipidaemia and thus to the pathogenesis of atherosclerosis and elevated risk of cardiovascular disease (Ross & Harker, 1976). Fatty acid oxidation leads to the generation of reactive oxygen species (ROS) in liver and oxidative stress (Sanyal, Campbell-Sargent, *et al.* 2001) which contribute to hepatotoxicity and exacerbation of local inflammatory pathways.

While obesity is a risk factor for hepatic steatosis, accumulation of liver fat is proportional to the degree of insulin resistance, independent of body mass index or total body fat content (Korenblat, Fabbrini, *et al.* 2008). Genetic factors may also influence hepatic fat accumulation independent of body weight, such as variants of patatin-like phospholipase domain-containing protein 3 (PNPLA3) (Romeo, Kozlitina, *et al.* 2008). Bariatric surgery and

weight loss have been shown to reduce liver fat and quickly improve hepatic insulin resistance (Petersen, Dufour, *et al.* 2005; Vitola, Deivanayagam, *et al.* 2009; Lim, Hollingsworth, *et al.* 2011; Rossi, Fantin, *et al.* 2012). Hepatic lipid content can also be reduced by increased exercise, independent of an effect on body weight (Hallsworth, Fattakhova, *et al.* 2011). Regulation of liver fat is therefore not simply a function of total body fat but has a specific role in regulation of hepatic glucose production and insulin sensitivity.

In skeletal muscle, there is similar accumulation of excess lipid in obese diabetic subjects (Goodpaster, Thierault, *et al.* 2000). This may be due to reduced fatty acid oxidation in extreme obesity, but other mechanisms may be involved at earlier stages (Hulver, Berggren, *et al.* 2002). Similar to the situation in liver, there is generation of harmful lipid species which may interfere with insulin signalling in myocytes (Eckardt, Taube, *et al.* 2011). This includes species such as ceramide, which can reduce insulin-stimulated Akt phosphorylation (Adams, Pratipanawatr, *et al.* 2004). Similarly, exposure of myocytes to excess saturated fatty acids leads to activation of PKC θ and impaired insulin signalling (Griffin, Marcucci, *et al.* 1999) as well as enhancing IL-6 secretion via nuclear factor- κ B (Weigert, Brodbeck, *et al.* 2004). However, IL-6 may also have a role in increasing glucose uptake and fatty acid oxidation in muscle via an adenosine monophosphate kinase (AMPK) pathway (Glund, Deshmukh, *et al.* 2007)

In pancreas, there is initially a functional upregulation of beta cell activity, contributing to hyperinsulinaemia, (Ferrannini, Camastra, *et al.* 2004) which can be mimicked by treatment of isolated islets with free fatty acids (Milburn, Hirose, *et al.* 1995). In the long term however, the lipotoxic effect of

lipid accumulation in beta cells lead to reduced beta cell function, contributing to the pathogenesis of type II diabetes (Lee, Hirose, *et al.* 1994). This process can be ameliorated by treatment with thiazolidinediones, which reduced islet cell triglyceride accumulation (Shimabukuro, Zhou, *et al.* 1997).

The multisystem effects of obesity stem from a vicious cycle of disordered metabolic regulation which contributes to upregulation of inflammatory pathways, which in turn, hinder normal metabolic regulation. The end outcomes of such processes are reflected in the pathologies associated with obesity: coronary vascular disease, diabetes, cerebrovascular disease and hypertension, and involves dysregulation of multiple cellular regulatory pathways.

1.3.3. Variable relationship between Adiposity and Insulin Resistance

It is worth noting that obesity is not exclusively associated with an adverse metabolic phenotype (Ferannini, Natali, *et al.* 1997; Bonora, Kiechl, *et al.* 1998; Karelis, St-Pierre, *et al.* 2004), and that up to 20% of obese patients have a large fat mass but normal insulin, normal insulin sensitivity high HDL cholesterol and low triglycerides: a metabolically healthy obese (MHO) phenotype. A large cohort study examining pooled data from over 1100 HEC studies conducted in Europe defined insulin resistance as the lowest decile of insulin sensitivity in the lean population, and showed that while the obese group as a whole were more insulin resistant, IR was less prevalent in the obese group than expected, occurring in only 26% of the obese group (Ferannini, Natali, *et al.* 1997). Insulin sensitivity was inversely correlated with BMI, but was still present in only 60% of subjects with BMI >30kg/m². However, it should also be noted that the relative risk of the MHO phenotype is not the same as lean and metabolically healthy; MHO subjects have evidence of vascular abnormalities and early atherosclerotic changes (Karelis, St-Pierre, *et al.* 2004).

Similarly, within the lean population there was significant variability in insulin sensitivity, as well as a subset of the population with an adverse metabolic profile, described as metabolically obese but normal weight (MONW). These individuals tended to have normal BMI but higher total fat mass, both subcutaneous and visceral, as well as an increased percentage body fat. Physical activity energy expenditure tended to be lower though similar CV fitness and low physical activity appears to play a major role in the development of this phenotype (Karelis, St-Pierre, *et al.* 2004).

The MHO phenotype is commonly seen in women with early onset of obesity (<20 years of age), and is associated with significantly reduced visceral fat mass (~50%), despite equivalent overall fat masses (Karelis, St-Pierre, *et al.* 2004). In a German study of 300 obese subjects with risk factors for insulin resistance (previous IGT or GDM), Stefan *et al* observed that while insulin resistance strongly correlated with BMI and measures of subcutaneous fat, visceral fat, WHR and total body fat, insulin-sensitive obese had significantly less intramyocellular and hepatic fat (Stefan, Kantartzis, *et al.* 2008). Visceral fat tended to be greater in obese IS subjects compared with obese IR subjects, but this was not statistically significant. These authors suggest that ectopic lipid distribution is therefore a better indicator of insulin resistance than visceral fat accumulation.

The existence of an MHO and a MONW phenotype is of significance for 2 reasons. Firstly, study data must be interpreted with caution, as up to a fifth of an obese group may have a metabolic profile differing from the remainder of the cohort. Secondly, the clinical management of such patients should be carefully considered, particularly issues such as intensive weight loss despite a benign metabolic profile.

1.4. Adaptation of maternal metabolism in pregnancy

The energy requirements of human pregnancy require a significant adaptation of maternal metabolism. Early pregnancy is characterised by relative lipid accretion, laying down energy stores against the upcoming needs of late pregnancy, when fetal growth is greatest, and the post-partum demands of breast-feeding ensue. This early pregnancy phase involves maintenance of insulin sensitivity (Catalano, Huston *et al.* 1999), with consequent lipid accretion (Franz, 1978). In contrast, late pregnancy is characterised by the progressive development of insulin resistance, first described by Burt (Burt., 1956). The mechanisms are not fully understood, but a role for placental hormones is likely, including human placental lactogen, and placenta-derived leptin. The development of insulin resistance in adipose tissue has the effect of releasing the lipolytic cascade from inhibition, promoting release of maternal free fatty acids and glycerol as gluconeogenic substrates, partitioning fetal energy requirements, supplied by dietary glucose, from those of the mother.

1.4.1. Insulin sensitivity in pregnancy

Insulin sensitivity in normal pregnancy is initially maintained at pre-gravid levels, or slightly increased, followed by progressive development of systemic maternal insulin resistance (Spellacy, Goaetz, *et al.* 1965; Catalano, Tyzbir, *et al.* 1991). In the third trimester, there is compensatory hyperinsulinaemia with enhanced first- and second-phase insulin response to glucose (Buchanan, Metzger, *et al.* 1990; Catalano, Drago, *et al.* 1998), as well as increased clearance of insulin (Catalano, Drago, *et al.* 1998). There is increased basal hepatic glucose production and a 40-60% decrease in insulin-sensitive glucose disposal (Catalano, Huston, *et al.* 1999). Development of third trimester insulin resistance is thought to be important for normal fetal growth; insulin sensitivity in late pregnancy correlates inversely with both placental weight and birthweight (Catalano, Drago, *et al.* 1995). In sheep, there is increased insulin sensitivity and glucose uptake in association with placental insufficiency and intra-uterine growth restriction (Limesand, Rozance, *et al.* 2007).

Assessment of insulin sensitivity in pregnancy can be made using the same indices as are applied to the non-pregnant population, ie. fasting measures such as HOMA-IR or QUICKI or dynamic assessments such as during oral glucose tolerance testing or the hyperinsulinaemic euglycaemic clamp (HEC). Different approaches are required depending on the context, whether as part of a research study, or as a clinically useful test applied to a population. In pregnancy insulin sensitivity as derived from sampling during 2 hour OGTT, as well as indices such as HOMA-IR and QUICKI have all been validated against insulin sensitivity measured during

hyperinsulinaemic euglycaemic clamp conditions and correlate closely (Kirwan, Huston-Presley, *et al.* 2001).

During clamp studies, insulin sensitivity can be expressed as M, which is the glucose disposal rate, derived from mean glucose infusion rate during steady state conditions in mg/kg/min. In pregnancy, glucose uptake by the fetoplacental unit occurs by facilitated diffusion down a concentration gradient. Theoretically, the fetoplacental unit acts as a glucose 'sink' in pregnancy, particularly in the third trimester when fetal energy requirements are greatest. This could lead to potential overestimation of insulin sensitivity in the third trimester, with an increased contribution of the fetoplacental unit to apparent maternal glucose disposal. While estimates of the decrease in insulin sensitivity with advancing gestation have been approximately 40-60% (Catalano, Tyzbir, *et al.* 1993; Catalano, Huston, *et al.* 1999), the true decrement in insulin sensitivity could therefore be greater than this. Assessment of the rate of specific glucose uptake by the fetus and placenta is difficult to measure *in vivo*; in rats, there is some evidence to suggest that placental glucose transport is not directly sensitive to maternal insulin concentrations (Norris, Wang, *et al.* 2011), and although glucose uptake is sensitive to maternal hyperglycaemia, under clamped euglycaemic conditions, fetal uptake may therefore not make a significant contribution to clamp-derived M-values. With these potential limitations in mind, euglycaemic clamp studies remain the gold standard method of assessment of insulin sensitivity in both pregnant and non-pregnant populations.

Skeletal muscle is a major site of insulin-stimulated glucose uptake. In pregnancy, this is significantly reduced by approximately 30-40% by the

third trimester (Friedman, Ishizuka, *et al.* 1999). This is associated with decreased IRS1 but increased IRS2 protein phosphorylation (Friedman, Ishizuka, *et al.* 1999). Protein levels of the GLUT4 transporter in skeletal muscle were not affected by pregnancy (Garvery, Maianu, *et al.* 1992).

The mechanisms driving such adaptations are not clear but are likely to involve pregnancy-specific hormones, as well as co-opting existing maternal pathways regulating maternal metabolism. Two of the main pregnancy-specific hormones include human placental lactogen (hPL) and human placental growth hormone (hPGH). Both these hormones are synthesized and secreted by placenta.

hPL induces profound systemic insulin resistance when administered intravenously, with impaired peripheral glucose utilisation, despite increased plasma insulin response to glucose (Beck & Daughaday, 1967). hPL acts through both human growth hormone (hGH) and human prolactin (hPRL) receptors, which account for its somatotrophic and lactogenic activities. The signalling pathways employed involve phosphorylation and activation of the JAK-STAT pathways (Takeda, Kurachi, *et al.* 1997). hPL also acts to increase the mass of pancreatic islets and improve insulin secretion (Lombardo, De Angelis, *et al.* 2011).

hPGH is secreted by syncytiotrophoblast and replaces maternal pituitary GH by week 20-24. hPGH is also able to induce insulin resistance, particularly in skeletal muscle; the mechanism is thought to be decreased PI3-kinase activity via upregulation of the p85 α regulatory subunit (Barbour, Shao, *et al.* 2002; Barbour, Shao, *et al.* 2004).

Other hormones involved in the regulation of insulin resistance in pregnancy include adipokines, inflammatory cytokines and the sex steroids, with many parallels with the mechanisms employed in obesity.

Adiponectin may have a role in insulin resistance of pregnancy. In non-pregnant individuals, adiponectin usually has an insulin-sensitising action (Section 1.2.2). In normal weight pregnant women, both plasma levels and white adipose tissue mRNA levels are significantly reduced by mid-third trimester (Fuglsang, Skærbæk, *et al.* 2006; Catalano, Hoegh, *et al.* 2006; Nien, Mazaki-Tovi, *et al.* 2007). Compared with post-partum, adiponectin levels are elevated during pregnancy but gradually decline (Mazaki-Tovi, Kanety, *et al.* 2007). In contrast, in overweight women, adiponectin is suppressed compared to lean women but does not show a further decrease in response to pregnancy (Nien, Mazaki-Tovi, *et al.* 2007). Whether decreased adiponectin in pregnancy is a direct response to pregnancy or a simply a function of increased fat mass during pregnancy is not clear although adiponectin levels appear to correlate closely with both fasting insulin and insulin-mediated glucose disposal in early pregnancy (Catalano, Hoegh, *et al.* 2007). In weight-matched subjects with gestational diabetes, adiponectin is further reduced compared to non-diabetic subjects, including prior to onset of gestational diabetes (Williams, Qui, *et al.* 2004; Retnakaran, Hanley, *et al.* 2004). In pregnancy, placenta is a source of adiponectin (Caminos, Nogueiras, *et al.* 2005; Lappas, Yee, *et al.* 2005; Chen, Tan, *et al.* 2006) so it is probable that there is a specific role for adiponectin rather than a secondary change in expression following other adaptations of maternal metabolism. This may

include regulation of placental response to insulin and placental amino acid transport (Jones, Jansson, *et al.* 2010).

In contrast to adiponectin, circulating levels of resistin are increased in pregnancy, particularly in the third trimester where insulin resistance is maximal (Chen, Dong, *et al.* 2005; Nien, Mazaki-Tovi, *et al.* 2007b). Resistin is implicated in the development of insulin resistance in obesity and has both insulin-antagonising as well as pro-inflammatory activities (Section 1.3.1.2). Resistin is expressed by placenta, with greater mRNA levels in trophoblast at term compared with first trimester (Yura, Sagawa, *et al.* 2003) whereas white adipose tissue resistin mRNA levels did not appear to change with pregnancy, indicating a placenta-specific source of pregnancy-induced increase in resistin (Yura, Sagawa, *et al.* 2003).

The placenta is also a source of leptin (Matsuzaki, Ogawa, *et al.* 1997) and plasma levels increase with gestation (Sattar, Greer, *et al.* 1998; Helland, Reseland, *et al.* 1998; Melczer, Bánhidý, *et al.* 2003). Increases in plasma leptin have been observed prior to an increase in body weight, suggesting that changes in circulating leptin are not simply secondary to an increase in fat mass (Henson & Castracane, 2006). However, longitudinal changes in leptin have been shown to correlate with insulin sensitivity in some studies but not others (Laivuori, Kaaja, *et al.* 2000; Kirwan, Haugel-De Mouzon, *et al.* 2002; McLachlan, O'Neal, *et al.* 2006) and it may be that the main role of leptin is in another capacity, such as regulation of fetal growth and placental function (Hellan, Reseland, *et al.* 1998; Melczer, Bánhidý, *et al.* 2003).

Placenta is also a source of the pro-inflammatory cytokines TNF α (Chen, Yang, *et al.*, 1991) and IL-6 (Kameda, Matsuzaki, *et al.* 1990). Both these cytokines have been implicated in the development of insulin resistance associated with obesity (Section 1.3.1.1) but may also be important in determining the insulin resistance of normal pregnancy. In particular, TNF α has been inversely correlated with maternal insulin sensitivity in normal- and over-weight glucose tolerant pregnant women and can be used to predict changes in insulin sensitivity (Kirwan, Haugel-De Mouzon, *et al.* 2002).

Of other maternal hormones, the sex steroids oestrogen and progesterone also have a role in influencing maternal insulin regulation, as do maternal pituitary hormones, such as prolactin. Oestrogens (oestradiol, oestrone and 17 β -oestradiol) are increased in pregnancy, initially synthesised by corpus luteum and subsequently by placenta, and may have a role in the adaptation of the pancreas to the requirements of pregnancy as well as a direct effect on peripheral glucose utilisation via influencing skeletal muscle GLUT 4 expression (Nadal, Alonso-Magdalena, *et al.* 2009; Barros, Morani, *et al.* 2008). Prolactin may also influence β -cell mass and function, including increased insulin secretion and may be essential for normal glucose homeostasis in pregnancy (Brelje, Bhagroo, *et al.* 2008; Huang, Snider, *et al.* 2009).

In summary, adaptation and regulation of maternal insulin sensitivity in normal pregnancy is a complex process, likely originating from tissues of placenta/fetal origin but also co-opting maternal pituitary and ovarian hormones as well as adipokines.

1.4.2. Enhanced third trimester lipolysis

1.4.2.1. Assessment of lipolysis in pregnancy

Demonstration of enhanced stimulated lipolysis in isolated adipocytes from tissue of late pregnancy was first demonstrated in the late 1970s. In Wistar rats, while basal rates of lipolysis in isolated subcutaneous adipocytes were not significantly different between pregnant and non-pregnant, noradrenaline-stimulated lipolysis was enhanced in late pregnancy compared with lactation; this effect is lost at the time of parturition (Aitchison, Clegg, *et al*, 1982). In contrast, in omental adipocytes in sheep increased basal and isoprenaline-stimulated rates of lipolysis were observed towards the end of pregnancy and during lactation (Guesnet, Massoud, *et al*. 1987). In humans, basal as well as adrenaline-stimulated lipolysis is elevated in late compared with early pregnancy (Elliott, 1975) although Coltart and Williams showed inhibition of isoprenaline-induced lipolysis in whole subcutaneous adipose by insulin (Coltart & Williams, 1976). Others have also described normal basal but increased noradrenaline-induced lipolysis in abdominal subcutaneous adipose in early pregnancy compared with non-pregnant women (Rebuffé-Scrive, Enk, *et al*. 1984).

Previously, the presence of elevated circulating lipids or fatty acids had been accepted as evidence of active lipolysis. This may not be an accurate representation of the true rate of lipolysis due to re-uptake and re-esterification of triglyceride by adipocytes, muscle and liver and measurement of release of glycerol is likely to be more representative. This can be achieved by the use of stable isotope tracers such as D₂-glycerol. A study of lean women in the third trimester showed enhanced lipolysis along

with elevated endogenous glucose production (Diderholm, Stridsberg, *et al.*, 2005). A previous study of overweight and obese women showed that although basal lipolysis did not change with gestation or post-partum, that the ability of insulin to suppress lipolysis was significantly reduced in the third trimester (Sivan, Homko, *et al.*, 1999). It is possible that this phenomenon is essential for normal fetal growth in late pregnancy; as in women with IUGR pregnancies, third trimester lipolysis is reduced (Diderholm, Stridsberg, *et al.*, 2006).

1.4.2.2. Mechanisms of enhanced lipolysis

In terms of altered adipose tissue function in pregnancy, many of the mechanisms are common to those driving altered insulin resistance, resulting in freedom of the lipolytic pathway from suppression by insulin. However, some pregnancy-associated hormones also have direct lipolytic activity. Lipolysis is stimulated by hPL in adipose tissue *ex vivo* (Strange and Swyer, 1974), and has a greater stimulatory effect in tissue from pregnant than non-pregnant women (Williams and Coltart, 1978). However, the situation *in vivo* is less clear, as hPL can both stimulate lipolysis, an anti-insulin action, but also promotes insulin secretion in response to glucose by pancreatic beta-cells (Lombardo, De Angelis, *et al.* 2011).

Studies by Kirwan *et al* showed a strong correlation between circulating maternal TNF α and insulin resistance, a novel finding demonstrating that it is not only the classical regulators of lipid metabolism which play a role in the metabolic adaptations of pregnancy (Kirwan, Haugel-De Mouzon, *et al.* 2002). Altered adrenoceptor function in the third trimester may also have a role in regulation of lipolysis at this stage, with a modified balance between

alpha and beta-adrenoceptors, although this was described in a non-primate mammalian model (Bousquet-Mélou, Muñoz, *et al.* 1999).

1.5. Consequences of maternal obesity in pregnancy

1.5.1. Impact of maternal obesity on the mother

1.5.1.1. Fat distribution in pregnancy

Weight gain in pregnancy is highly variable; in lean women, average weight gain is approximately 10-12kg, secondary to gains in several compartments: approximately 3-4kg from fetus, 0.5kg from placenta, ~0.8kg amniotic fluid, ~3kg adipose tissue, 1.2kg blood, ~1.0 kg uterus and ~1.5kg extracellular extravascular fluid (Hytten & Chamberlain, 1991). Weight gain is predominantly subcutaneous, largely in trunk and thigh (Sohlström, Wahlund, *et al.* 1993; Sohlström and Forsum, E. 1995; Stevens-Simon, Thureen, *et al.* 2001). In obese women, gestational weight gain tends to be less than that of lean women (Soltani & Fraser, 2000; Ehrenberg, Huston-Presley, *et al.* 2003). However, obese women tend to accumulate more central weight gain compared to lean women (Soltani & Fraser, 2000). In 2009, the Institute of Medicine issued recommendations on gestational weight gain for women of differing pre-pregnancy BMI: 11.5 – 16 kg for women with BMI 18.5 – 24.9 kg/m², with smaller recommended weight gain in higher BMI groups, eg 5 – 9kg in patients with BMI > 30kg/m². In women gaining more than the recommended amount of weight, there was an associated increase in adverse outcomes (Crane, White, *et al.* 2009)

1.5.1.2. Maternal insulin & glucose regulation

In normal pregnancy there is progressive development of insulin resistance with compensatory hyperinsulinaemia (Spellacy & Goetz. 1963). In women who do not develop this compensatory hyperinsulinaemia, gestational diabetes can develop. Beta cell function is impaired, with reduced insulin response to glucose compared to women without GDM (Homko, Sivan *et al.*, 2001). Obesity is a risk factor for the development of GDM (Chu, Callaghan, *et al.* 2007) and likely reflects a reduced ability to mount a compensatory beta-cell response to pregnancy-induced insulin resistance (Qvigstad, Voldner, *et al.* 2010). The importance of glycaemic control in pregnancy was highlighted by the HAPO study, which has shown that maternal glucose levels are strongly correlated with pregnancy outcome including birthweight, even where maternal glucose levels fall below diagnostic criteria for gestational diabetes (Metzger, Lowe, *et al.* 2008).

1.5.1.3. Maternal vascular endothelial function

An association between obesity and increased incidence of hypertensive disorders in pregnancy has long been recognised (Fisher, & Frey, 1958). This includes increased incidence of pre-gravid chronic hypertension (Seely & Ecker, 2011) as well as development of pregnancy induced hypertension and pre-eclampsia (Eskenazi, Fenster *et al.*, 1991; Bianco, Smilen *et al.* 1998; Duckitt & Harrington, 2005; Jensen, Ovesen, *et al.* 2005). The risk of pre-eclampsia in a patient with BMI >35kg/m² is approximately double that of a normal weight women (Duckitt & Harrington, 2005).

Potential mechanisms by which obesity leads to an increase in hypertensive disorders in pregnancy include a detrimental effect on endothelial function, inflammation, dyslipidaemia and oxidative stress. Markers of endothelial dysfunction and inflammation are altered in pre-eclampsia, including nitric oxide (Choi, Im *et al.* 2002), sFlt-1 (Maynard, Min, *et al.* 2003), interleukin 6 (Greer, Lyall, *et al.* 1994) and VEGF (Lyall, Greer, *et al.* 1997). Obesity also predisposes to a pro-inflammatory environment with increased oxidative stress and dyslipidaemia and thus may contribute to a cycle of pathogenic changes that exacerbate development of conditions such as pre-eclampsia. In obese pregnant women, there is persistence of a pro-inflammatory environment compared with lean women as well as vascular dysfunction with altered endothelial-dependent and –independent relaxation (Ramsay, Ferrell, *et al.* 2002). It may be that each of these pathways feeds into a common mechanism lowering the threshold for the development of conditions such as pre-eclampsia.

1.5.1.4. Effects on parturition

Obese women are more likely to require induction of labour for prolonged pregnancy as well as instrumental and operative delivery (Sebire, Jolly, *et al.* 2001; Arrowsmith, Wray *et al.* 2011). They are also at increased risk of post-partum haemorrhage, particularly atonic haemorrhage (Sebire, Jolly, *et al.* 2001; Blomberg, M. 2011). These outcomes can be linked by ineffectiveness of myometrial contractility. The effect of obesity on the myometrium is not clear. There is some evidence for reduced inherent contractility of isolated myometrium from obese subjects (Zhang, Bricker, *et al.* 2007) although other studies have not shown any relationship between BMI and spontaneous contractility (Higgins, Martin, *et al.* 2009).

1.5.2. Impact of maternal obesity on the fetus

Maternal obesity has a number of influences on the fetus. At the very early stages in pregnancy, there is an increased risk of miscarriage (Lashen, Fear *et al.*, 2004) and congenital anomaly (Rasmussen, Chu, *et al.* 2008; Stothard, Tennant, *et al.*, 2009; Mills, Troendle, *et al.* 2010). Later on in pregnancy, there is an association with increased birthweight (Garn and Pesick, 1982), with associated morbidity secondary to delivery complications such as shoulder dystocia. There is a reduction in the number of low birthweight offspring, although some studies have reported a paradoxical increase in very low birth weight offspring, possibly secondary to placental dysfunction (McDonald, Han, *et al.* 2010). However, this may be confounded by the increased incidence of preterm deliveries (Madan, Chen, *et al.* 2010). The incidence of preterm birth is again confounded by an increased rate of induced preterm delivery for maternal indications; there is an overall reduction in spontaneous preterm labour (Hendler, Goldenberg *et al.* 2004).

The effect of obesity on fetal nutrient supply and birthweight is contentious. In healthy pregnancy, transport of nutrients to the fetus relies on transfer from maternal to fetal circulation across the placenta. In the case of some nutrients such as glucose, this occurs by diffusion along a concentration gradient from mother to fetus, facilitated by relative maternal hyperglycaemia (Chinard, Danesino *et al.*, 1956). In the context maternal diabetes, excess supply of glucose to the fetus with a compensatory fetal hyperinsulinaemia is thought to account for fetal macrosomia in such pregnancies (Skyler, O'Sullivan, *et al.* 1980). Amino acid transport on the other hand relies on active transport via specific transport proteins against a concentration gradient (Cleal & Lewis, 2008). In obese mice, there is upregulation of placental nutrient transport, with increased expression of

glucose transporter (GLUT) 1 and sodium-coupled amino acid transporter (SNAT) 2 as well as increased offspring birthweight (Jones, Woollett, *et al.* 2008). In sheep, maternal obesity also increases expression of some placental fatty acid transporters, as well as circulating fetal triglyceride and birthweight (Zhu, Ma, *et al.* 2010). In humans, there is some evidence for alteration of placental fatty acid transport in the context of maternal obesity, but this was not reflected in fetal hypertriglyceridaemia, elevated free fatty acids or greater birthweight (Dubé, Gravel, *et al.* 2012).

Of increasing importance is the impact of the maternal metabolic environment on long term offspring health. At birth, there is already evidence of altered fetal metabolism including altered adipokine levels and insulin resistance (Catalano, Presley, *et al.* 2009). Similarly, while birthweight is elevated, this is manifested in increased adiposity of the neonate, with elevated adiposity of offspring of obese mothers (Sewell, Huston-Presley, *et al.* 2006).

Later on in life, higher birthweight has been associated with increased risk of childhood and adolescent obesity, as well as adult obesity (Curhan, Willet, *et al.*, 1996; Whitaker, 2004). The relationships between birthweight and maternal metabolism and later life health are complex and it is difficult to dissect out the effects of genetics, in utero influences and later environmental effects. Low birthweight is also associated with later life central obesity (Loos, Beunen, *et al.* 2001; Kuh, Hardy, *et al.*, 2002), hypertension, diabetes and cardiovascular disease (Curhan, Willet, *et al.*, 1996), although this appears to be most strongly associated where there is co-existing obesity (Frankel, Elwood, *et al.* 1996).

In animal studies, maternal nutritional status has been shown to influence offspring body composition and metabolic characteristics although specific effects have been variable, reflecting the animal model used as well as the exact composition of diet and timing of exposure to the experimental diet. Maternal high fat feeding (45% fat) in Wistar rats has been observed to lead to reduced offspring birth weight but adult obesity (Howie, Sloboda *et al.*, 2008; Nivoit, Morens, *et al.*, 2009). Other rodent models have described alterations in offspring insulin levels, leptin levels, fat mass and blood pressure (Khan, Dekou, *et al.* 2004; Samuelsson, Matthews, *et al.* 2008). Maternal diet in pregnancy can influence later response to diet: offspring of Sprague-Dawley rats fed an obesogenic diet (75% carbohydrate) pre-conception had no difference in pup birth weight but postnatally, offspring weaned to high fat diet had a greater increase in body weight if they were offspring of obese compared to lean dams (Shankar, Harrell, *et al.* 2008). In smaller non-human primate models of maternal diet-induced obesity, there were similar trends towards increased adiposity of offspring of obese baboons (Farley, Tejero, *et al.* 2009) and obese macaques (McCurdy, Bishop, *et al.* 2009).

Mechanisms by which the maternal environment programmes fetal metabolism include effects on central regulation of appetite and feeding behaviours with altered expression of thalamic neurotransmitters (Chang, Gaysinskaya, *et al.*, 2008; Kirk, Samuelsson, *et al.*, 2009; Sullivan, Grayson, *et al.* 2010), as well as interaction between prenatal stress and glucocorticoid exposure (Drake & Walker, 2004). Peripherally, effects on adipocyte structure and function have been reported; in a murine model of diet induced obesity, there was evidence of adipocyte hypertrophy and altered expression of adrenoceptors, 11 β -HSD-1 and PPAR γ 2 at 3 months in offspring of high fat

fed compared with lean dams (Samuelsson, Matthews, *et al.* 2008). Similarly, in an ovine model, maternal overnutrition stimulates PPAR γ , LPL, adiponectin and leptin mRNA expression in perirenal fat of offspring, predisposing to lipogenesis (Muhlhausler, Duffield, *et al.* 2007).

It also becoming increasingly apparent that maternal obesity has an impact at the periconceptual stage; obesity has been shown to have an effect on oocyte quality. Normally maturing oocyte contain lipid droplets and there is some evidence in murine models that excess lipid accumulation in oocytes occurs in animals on a high fat fed diet, similar to ectopic lipid accumulation observed at other sites such as liver or skeletal muscle in obesity (Wu, Dunning, *et al.* 2010). Lipid accumulation is then associated with excess endometrial reticulum stress, generation of reactive oxygen species and mitochondrial dysfunction as well as impaired ability to maintain a pregnancy (Wu, Dunning, *et al.* 2010; Igosheva, Abramov, *et al.* 2010). This may contribute to reduced fertility observed in obese women, but also to periconceptual programming of embryos via suboptimal maternal mitochondrial function.

Not only are there direct effects of maternal obesity on offspring metabolism, but these effects also appear to persist to subsequent generations, potentially through epigenetic mechanisms (Drake and Walker, 2004; Waterland, Travisano, *et al.* 2008; Davis, McGonagle, *et al.* 2008; Aagaard-Tillery, Grive, *et al.* 2008; Li, Huang, *et al.* 2012).

It is therefore clear that the maternal environment is not only crucial for the normal development of offspring metabolic regulation but that there are potential significant implications for the health of future generations.

1.6. Summary

This thesis addresses aspects of maternal metabolism in apparently healthy obese pregnant women. While there is an abundance of epidemiological data describing the increased risk of adverse outcomes for mother and offspring in the context of obesity in pregnancy, there is less known about the underlying mechanisms to account for these outcomes. The complex endocrine regulatory activities of adipose tissue are only beginning to be described in the relatively recent literature, as are the cellular perturbations observed in obesity in non-pregnant individuals.

The long-term effects of obesity contribute to a vicious cycle of obesity and further ill health in subsequent generations. At present, there are few effective therapies and no specific pharmacological targets. The interactions between the normal physiological adaptations of pregnancy and those associated with pre-gravid obesity, outwith the context of co-morbidities such as gestational diabetes or vascular disease, are the focus of this thesis.

1.7. Hypothesis and Aims

This project was designed with the aims of initially describing features of intermediary metabolism in healthy obese pregnant women and how these diverge from those of normal weight women, particularly with respect to characteristics of adipose tissue.

We hypothesised that:

1. Increased severity of obesity is associated with exaggerated insulin resistance secondary to interaction between pre-existing obesity and the metabolic adaptations of pregnancy compared with less obese and lean women.
2. That this exaggerated insulin resistance is associated with adaptation of adipose tissue function in terms of expression of adipokines and inflammatory cytokines. Specifically, that expression of adipokines and inflammatory cytokines is altered in pregnant compared with non-pregnant women, and further exaggerated in obese compared with lean pregnant women.
3. That there is enhanced adipose tissue lipolysis compared with lean pregnant controls.
4. That this state of insulin resistance and enhanced lipolysis is associated with ectopic lipid accretion in liver and skeletal muscle.

2.1. Patient Recruitment**2.1.1. Ethical approvals**

Ethical approval for this study was obtained from the Lothian Research Ethics Committee. Three ethical approvals relate to this thesis:

1. LREC 07/S1103/25. 'The effect of maternal weight on the fetomaternal unit in pregnancy'. Chief investigator, Dr Fiona Denison.
2. LREC 09/S1103/6. 'Altered Metabolic Processes in Obese Pregnant Women' (AMPOP study). Chief investigator, Professor Jane Norman; principle investigator, Dr Sarah Barr.
3. LREC 08/S1101/39. 'Hormones in Pregnancy'. Chief investigator, Dr Rebecca Reynolds.

Also applicable is the following approval:

1. West of Scotland REC4, 09/S0704/3, 'Edinburgh Reproductive Tissue BioBank' (ERTBB). Chief investigator, Dr Simon Riley.

2.1.2. Recruitment process/Metabolic Clinic

For studies in tissues obtained from term pregnancies (Chapter 3), tissue (blood, subcutaneous adipose, omental adipose and placenta) was obtained from healthy women undergoing elective caesarean section at the Royal Infirmary of Edinburgh and who had consented to storage and use of tissues in the ERTBB. Specific inclusion/exclusion criteria are detailed in the relevant chapters.

For longitudinal studies in pregnancy, obese women were recruited from the Metabolic Antenatal Clinic at the Royal Infirmary of Edinburgh. This is a pan-Lothian high risk antenatal clinic for women with a booking BMI greater than 40kg/m². Normal weight (BMI 20-25 kg/m²) 'control' women were recruited from routine antenatal clinics in Edinburgh.

Non-pregnant participants were recruited as volunteers responding to advertisements.

2.1.3. Oral Glucose Tolerance Testing

When women undertook oral glucose tolerance testing, the following standard method was used. Following an overnight fast, participants attended the Royal Infirmary of Edinburgh at 8am. Fasting venous blood samples were obtained from a peripheral vein. Glucose (75g) was administered as oral Lucozade Energy Original (410ml of 70kCal/100ml formulation) (GlaxoSmithKline). A second venous sample was obtained 2 hours after glucose administration. Gestational diabetes was diagnosed on the basis of either fasting glucose > 5.5 mmol/L or 2 hour glucose > 8.9 mmol/L (Scottish Intercollegiate Guideline Network (SIGN) 55 criteria).

2.2. Tissue collection

2.2.1. Blood sampling

Whole blood was collected from a peripheral vein into the appropriate container (Sarstedt Monovette ®, UK) and transported on ice. Plasma or serum was separated within 30 minutes of collection by centrifugation at 12000rpm for 10minutes at 4°C. Blood fractions were stored at -80°C until analysis.

Blood collected for plasma was anti-coagulated with either sodium EDTA or lithium heparin. Blood collected for serum was collected into either a plain container or one containing a separation polyacrylic ester gel. For glucose determination, samples were collected into tubes containing fluoride (1mg/ml blood) and EDTA.

2.2.2. Third trimester adipose tissue biopsy

2.2.2.1. Site of biopsies

Adipose tissue biopsies were obtained from women undergoing elective caesarean section. Subcutaneous adipose tissue was excised by diathermy or sharp dissection from the abdominal adipose depot at the site of skin incision in the lower abdomen. Visceral adipose tissue was obtained from the omentum by diathermy or sharp dissection.

2.2.2.2. Tissue handling

Any damaged tissue (identified macroscopically) was quickly excised from the sample. Tissue was divided into aliquots and placed immediately on dry ice and then stored at -80°C for later use.

2.2.3. Percutaneous adipose tissue biopsy

Subcutaneous adipose tissue was biopsied as part of the AMPOP study protocol (see Chapter 4). With the patient supine, an area of skin lateral to the umbilicus was cleaned with sterile solution (chlorhexidine) and draped. An area 3cm in diameter was infiltrated with 1% lidocaine without adrenaline. Using a 12G needle inserted laterally into the subcutaneous tissue, adipose tissue was aspirated into a sterile 50ml syringe under suction. Approximately 10ml sterile isotonic saline (supplier) was drawn up into the syringe, then tissue was rinsed over sterile wire mesh to remove excess blood. Visible blood clots were removed with sterile forceps. Aspirated tissue was placed in a sterile 2ml eppendorf and placed immediately on dry ice. Tissue was stored at -80°C until use. Up to three aspirations were carried out to obtain sufficient tissue.

In pregnant women, abdominal examination was carried out to determine fundal height prior to proceeding with the biopsy. When biopsies were carried out in the third trimester, left lateral tilt was applied to prevent aorto-caval compression.

2.3. Materials

2.3.1. Blood collection

- S-Monovette® blood collection system (Lithium Heparin, EDTA, fluoride and serum gel) Sarstedt Ltd., Leicester, UK

2.3.2. RNA extraction & cDNA synthesis

- RNeasy Lipid minikit Qiagen, Crawley, UK Cat. No. 74804
- RNase-free DNase I Qiagen, Crawley, UK Cat. No. 79254
- QIAzol Lysis Reagent Qiagen, Crawley, UK Cat. No. 79306
- Chloroform (>99%, PCR reagent) Sigma, Dorset, UK C7559-5VL
- Ethanol [for molecular biology] Sigma, Dorset, UK E7023-4X4L
- High capacity cDNA synthesis kit Applied Biosystems 4368816
- RNase inhibitor Applied Biosystems AM2682

2.3.3. Real time RT-PCR (Taqman®) Gene expression assays & solutions

- Cyclophilin A Applied Biosystems Hs99999904_m1
- FAS Applied Biosystems Hs01005622_m1

• ME1	Applied Biosystems	Hs00159110_m1
• RBP4	Applied Biosystems	Hs00924047_m1
• DGAT2	Applied Biosystems	Hs00261438_m1
• HSD17b2	Applied Biosystems	Hs00157993_m1
• PPARalpha	Applied Biosystems	Hs00947537_m1
• SCD1	Applied Biosystems	Hs01682761_m1
• 11 β -HSD1	Applied Biosystems	Hs00194153_m1
• LPL	Applied Biosystems	Hs00173425_m1
• HSL	Applied Biosystems	Hs00193510_m1
• ATGL	Applied Biosystems	Hs00982042_m1
• Leptin	Applied Biosystems	Hs00174877_m1
• Adiponectin	Applied Biosystems	Hs00605917_m1
• IL6 primers/probes	Applied Biosystems	Hs00985639_m1
• TNF α	Applied Biosystems	Hs00174128_m1
• MCP1	Applied Biosystems	Hs00234140_m1
• CD68	Applied Biosystems	Hs00154355_m1
• 18S	Applied Biosystems	4319413E
• TaqMan® Universal PR Mastermix	Applied Biosystems	4364338
• MicroAmp® reaction plates (96- and 384- well)	Applied Biosystems	4366932/4326270
• MicroAmp® optical Adhesive Film	Applied Biosystems	4360954

2.3.4. ELISA

- IL6 High Sensitivity ELISA R&D systems, UK HS600B
- TNF α High Sensitivity ELISA R&D systems, UK HSTA00D
- MCP1 ELISA RayBio, UK ELH-MCP1-001
- Leptin Quantikine ELISA R&D systems, UK DLP00
- Adiponectin Quantikine ELISA R&D systems, UK DRP300
- High-molecular weight (HMW) Adiponectin ELISA Millipore, St. Charles, USA EZHMTWA-64K
- Leptin ELISA RayBio, UK ELH-Leptin-001
- Adiponectin ELISA RayBio, UK ELH-ADIPONECTIN-001
- Insulin ELISA Mercodia, Sweden 10-1113-01
- NEFA assay Wako Diagnostics, USA

2.3.5. Tracer & Hyperinsulinaemic Euglycaemic Clamp

- Accu-Chek Blood Glucose Monitor Roche Diagnostics Ltd., Burgess Hill, UK
- 6,6-d₂-glucose Cambridge Isotopes, CK Gas Products, Hampshire, UK DLM-349-SP-40
- 1,1,2,3,3-d₅-glycerol Cambridge Isotopes, CK Gas Products, Hampshire, UK DLM-1229-SP-10
- Actrapid Insulin Novo Nordisk, Crawley, UK
- Glucose 20% Baxter, Berkshire, UK FKB0213B

2.3.6 Gas Chromatography –Mass spectrometry

- HPLC grade Water Fisher Scientific (Loughborough, UK)
- HPLC grade acetonitrile VWR (Lutterworth, UK)
- Heptane Rathburn (Walkerburn, UK)
- Pyridine Sigma, Dorset, UK
- Acetic anhydride Sigma, Dorset, UK
- Glucose Sigma, Dorset, UK
- Glycerol Sigma, Dorset, UK
- Methane, research grade BOC, Glasgow, UK

2.4.Molecular Biology

2.4.1. RNA extraction

RNA extraction was carried out from whole frozen adipose tissue using Qiagen RNeasy Lipid Mini kit as follows.

1. Tissue (100mg) was disrupted in 1ml Qiazol reagent using a tissue lyser and steel beads (TissueLyser, Qiagen, UK) for 3 x 3 minutes at 25Hz.
2. Tissue homogenate was then transferred to new tubes and incubated at room temperature for 15 minutes.
3. Chloroform (200 μ L) was added to each tube, shaken vigorously and incubated at room temperature for 2-3 minutes.
4. Samples were centrifuged at 12 000 x g for 15 minutes at 4°C and separated into layers.
5. The uppermost aqueous layer was transferred to clean tubes, taking care not to transfer solvent or protein material.
6. Ethanol (70%) was added (approximately 600 μ L, equivalent to the volume of aqueous layer obtained), vortexed and the mix applied to the RNeasy spin column in 2 x 700 μ L batches. Following centrifugation at 8000 x g at room temperature, RNA was separated by affinity to the membrane and eluate discarded.
7. On column DNaseI digestion was carried out (Qiagen); DNaseI was applied to the membranes for 15 minutes at room temperature to specifically degrade genomic DNA; columns were then washed by applying buffer RW1.

8. Columns were subsequently washed with buffer RPE and RNA was eluted from the column using 50 μ L RNase free water, followed by a second elution with eluate to increase the final concentration of RNA.

RNA concentration was then evaluated spectrophotometrically by Nanodrop. RNA purity was assessed using A260/280 ratio, ratios between 1.8-2.0 were satisfactory. RNA contamination with protein and solvent was assessed using A260/230 ratio, ratios between 2.0 and 2.2 were satisfactory. RNA was stored at -80°C until use.

2.4.2. cDNA synthesis

RNA (200-500ng) was reverse transcribed in 20 μ L reaction volume using cDNA synthesis kit (High capacity cDNA reverse transcription kit, Applied Biosystems, UK). Random primers were used (ABI, UK). Reverse transcriptase negative and template negative controls were also synthesized with each cDNA prep.

cDNA was synthesised using a GS1 thermal cycler(GStormTM), with a cycle of: 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes. cDNA was stored at -20°C until use.

cDNA synthesis reaction volumes are shown in Table 2.1.

Table 2.1. cDNA synthesis mixes and controls.

	1x reaction volume	RT negative	Template negative
500ng RNA	Make up to 10 μ L with DEPC water	Make up to 10 μ L with DEPC water	-
Random primers	2	2	2
dNTP	2	2	2
Reverse transcriptase	1	-	1
DEPC water	5	6	15
Final volume	20 μ L	20 μ L	20 μ L

2.4.3. Real time reverse-transcriptase PCR

2.4.3.1. Method

Relative messenger RNA expression was determined using quantitative real time PCR (qRT-PCR) (TaqMan®, Applied Biosystems) using pre-designed gene expression assays (Applied Biosystems, UK). Reactions were set up in 96- or 384-well MicroAmp® fast optical reaction plates containing 24µL Master Mix and 1µL cDNA (for 96-well plates; for 384 well plates, a 12µL reaction volume was used in the same proportions). Plates were sealed with an optical adhesive cover. Standard curves were generated using serial 2-fold dilutions of pooled cDNA and included in each assay; concentrations of unknowns are therefore expressed relative to all samples in the experiment. Reverse transcriptase negative (to exclude contamination by genomic DNA), template negative (to exclude contamination in the cDNA synthesis process) and water (to exclude contamination of the reagents) controls were included in each experiment; all samples were run in triplicate. The gene of interest and housekeeping genes were run in separate singleplex reactions from the same cDNA prep. The probe for the gene of interest was labelled with the reporter dye FAM; housekeeping genes were labelled with the reporter dye VIC; both probes used the quencher dye TAMRA at the 3' end. The PCR reaction was run on the ABI Prism® 7500 Sequence Detection system (Applied Biosystems) with the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 x cycles of 95°C for 15 seconds then 60°C for 60 seconds, 72°C for 10 minutes. Data was analysed using the programme SDS Version 2.1 (Applied Biosystems). Baseline was set manually 3-4 cycles prior to the earliest amplification. The threshold was set manually in the exponential portion of the amplification curve.

2.4.3.2. Data analysis

Relative quantification of transcript levels was determined using a standard curve method. Mean Ct (cycle threshold) of each standard was plotted against \log_{10} dilution factor and linear regression was used to fit a standard curve to the data. Acceptable standard curves had a slope between -3.1 and -3.5, with $r^2 > 0.97$. Unknown samples were diluted 1:20 or 1:40 for use. Mean Cts of unknowns were interpolated from the standard curve, deriving a \log_{10} relative dilution value (A) for that sample; this value was then exponentiated to the power of 10 to give the final relative concentration of RNA:

$$\text{relative concentration} = 10^A \times \text{sample dilution factor}$$

Finally relative transcript levels were normalised against expression of a housekeeping gene for subsequent analysis.

2.4.3.3. Housekeeping Gene Choice

18S (eukaryotic ribosomal RNA) (Hs99999901_s1) and cyclophilin A (Hs99999904_m1) were used as housekeeping genes.

18S was initially chosen for these experiments as it is commonly used in the literature, both in adipose tissue and in other tissues. It is highly abundant in most cell types, representing approximately 80% of total cellular RNA and codes for the small subunit of eukaryotic ribosomes. However, when undiluted and/or in high abundance 18S leads to template inhibition of the TaqMan reaction and unreliability of the standard curve at high concentrations. The 18S standard curve is not linear at high concentrations. A range from 1:2 to 1:1024 was used; the curve became linear at 1:32 therefore a

range from 1:32 to 1:1024 was used for the remainder of the experiments. As most of the patient samples were used initially diluted 1:20, the majority did not fall within the standard curve of 18S. Additionally, many of the target genes were expressed at a much lower frequency, and some, such as adipose triglyceride lipase and glucocorticoid receptor, become undetectable above 1:256 dilution. Diluting the samples to 1:40 appeared to allow sufficient dilution for 18S, without diluting out the target genes.

18S was suitable as a housekeeping gene as there was no correlation between 18S expression and BMI and it is therefore stable across the range of samples where target genes might be expected to vary. Ideally, more than one housekeeping gene should be employed (Gabrielsson, Olofsson, *et al.*, 2005). Cyclophilin A was used in later experiments; it has been used in adipose tissue by others in this department (Wake, Stimson *et al.*, 2007), and is a satisfactory choice for a second housekeeping gene in adipose tissue. One potential limitation of this as a housekeeping gene is that it may be upregulated in response to some inflammatory stimuli in a murine model (Nigro, Satoh, *et al.* 2010) and is reported as possibly affected by hyperglycaemia *in vitro* in one study (Ramachandran, Venugopal, *et al.* 2012). Both of these conditions may be associated with obesity. As none of the population studied in this thesis were diabetic and samples were obtained fasting, then hyperglycaemia is not likely to be a major consideration in this population. There is no reported direct association between obesity and levels of cyclophilin A. It is of greater practical use compared to 18S as transcript levels of cyclophilin A were in a similar range of abundance as the genes of interest, there were no issues with template inhibition at higher concentrations.

2.5. Enzyme-linked Immunosorbent Assay (ELISA)

Commercially available ELISA kits were used, according to manufacturers' instructions. Briefly, the basis for the ELISA assay is selective binding of the protein of interest in a sample to a specific monoclonal antibody which is anchored to the surface of a 96-well plate. Sample was applied to the pre-coated plate and incubated (2hours – overnight). The sample is then removed and the plate washed (typically, phosphate buffered saline with Tween detergent (PBS-T), commercially supplied wash buffer with each kit was used). This removes unbound protein, leaving the protein of interest bound to the plate. A secondary detection antibody is then applied. This is usually a second antibody specific for the protein of interest which has been biotinylated; the plate is washed again to remove unbound detection antibody. The protein of interest is now sandwiched between the coated primary antibody and the secondary detection antibody. The detection antibody is quantified by addition of a solution of horseradish peroxidase conjugated to streptavidin; streptavidin binds biotin tightly and specifically. Unbound streptavidin is washed off and a detection solution applied. The detection solution contains a molecule which releases a coloured dye in response to peroxidase activity, for example 3,3',5,5'-tetramethylbenzidine (TMB), which forms 3,3',5,5'-tetramethylbenzidine diimine which has a blue colour. This particular reaction is stopped by addition of sulphuric acid, leading to a colour change to yellow. Light absorption at 450nm is measured by spectrophotometry; absorption is proportional to the concentration of yellow species present, which is directly proportional to the concentration of the primary protein of interest in the sample.

Details of intra-assay and inter-assay co-efficients of variation for each assay are given in the relevant chapters.

2.6.AMPOP study: Gas Chromatography Mass Spectrometry (GCMS): Protocol

Gas Chromatography Mass Spectrometry (GCMS) is a technique to separate and quantify chemical species. Chromatography is initially employed to separate compounds of differing molecular mass in a mixture on the basis of their specific elution times as they pass along a column. Samples are dissolved in a volatile liquid which is injected into a column coated with thin layer of liquid (stationary phase); the samples are vaporised and carried through the column by an inert gas such as helium (mobile phase). Interactions between the different species and the stationary phase slow down movement of the compounds in the mixture through the column so that they are eluted at different times depending on their mass.

Species emerging from the column are then detected by mass spectrometry. This technique uses ionisation of each molecule as it leaves the column; ions then pass into the analyser, which measures the mass:charge ratio (m/z) and intensity. This data are displayed as a peak, with retention time on the x-axis; peak area is proportional to the quantity of that ion present. Specific compounds can be identified by their characteristic spectra.

For the quantification of many stable isotopomers in biological samples, samples must first be derivatised to stabilise them for analysis as the isotopomers alone are not suitable for direct analysis by GC-MS. Plasma samples were prepared for GCMS analysis by 96-well protein precipitation, derivatised to their acetates using a 1:1 (v/v) mix of pyridine and acetic anhydride.

The sample preparation protocol is as follows:

- i. Prepare standard solutions.
 - a. Glucose (5mg/mL), glycerol (0.025 mg/mL), d2-glucose (0.1mg/mL) and d-glycerol (0.025mg/ml) in HPLC water. Once made up, can be stored for 3-4 weeks only. Store in fridge.
 - b. Internal standard mix. $^{13}\text{C}_6$ Glucose (5 mg/mL) and butanetriol (0.25mg/mL) in HPLC water.
- ii. Prepare reagents.
 - a. Acetic anhydride (5%) in heptane. Freshly prepared on day of assay.
 - b. Pyridine: acetic anhydride (1:1, *v/v*). Freshly prepared on day of assay.
- iii. Set up collection plate (Strata Impact Protein Precipitation 2ml plates, Phenomenex, UK). In each run, 8 x enrichment samples, 9 x standard samples and 1 x blank sample are included.
- iv. Preparation of enrichment standard samples.
 - a. Use glass tubes.
 - b. Prepare as per table below:

Standard	0%	0.25%	0.5%	1%	2%	4%	8%	Tracer only
D2 glucose (μL)	0	1.25	2.5	5	10	20	40	40
Glucose (μL)	500	498.75	497.5	495	490	480	460	0
D5 glycerol (μL)	0	1.25	2.5	5	10	20	40	40
Glycerol (μL)	500	498.75	497.5	495	490	480	460	0
HPLC water (μL)	0	0	0	0	0	0	0	120

'Tracer only' can be prepared directly into the cartridge well.

a.v. Extraction

- Add 1ml acetonitrile to each cartridge well.
- Prepare standards directly into the relevant cartridge well as per the following tables:

Quantities Required:

	Blank	0	1	2	3	4	5	6	7	8	9
Amount Glucose (µg)	Blank	0	10	20	50	100	150	200	300	400	500
Amount Glycerol (µg)	Blank	0	0.005	0.01	0.25	0.5	0.75	1.0	1.5	2.0	2.5
Amount D2 Glucose (µg)	Blank	0	0.2	0.4	1.0	2.0	3.0	4.0	6.0	8.0	10.0
Amount D5 Glycerol (µg)	Blank	0	0.005	0.01	0.25	0.5	0.75	1.0	1.5	2.0	2.5



Volumes of Stock Solutions Required (µL):

	Blank	0	1	2	3	4	5	6	7	8	9
Glucose 5mg/ml + Glycerol 0.025mg/ml	0	0	2	4	10	20	30	40	60	80	100
D2 glucose 0.1mg/ml + D5 glycerol 0.025mg/ml	0	0	2	4	10	20	30	40	60	80	100
HPLC water (µl)	200	200	196	192	180	160	140	120	80	40	0
Final Volume in cartridge (µL)	200	200	200	200	200	200	200	200	200	200	200

- c. Add 200 μ L of unknown plasma sample to separate wells.
 - d. To wells containing unknown samples and standard samples, add 25 μ L Internal Standard mix ($^{13}\text{C}_6$ Glucose and butanetriol as above).
 - e. Add 200 μ L of enrichment standards as prepared above to separate wells. No internal standard mix is required for these wells.
 - f. Wells are thus prepared as follows
 - Standards Standard + Internal Standard mix x 9
 - Enrichment Enrichment standard alone x 8
 - Blank 200 μ L HPLC grade water
 - Unknowns Plasma sample + Internal Standard mix
 - g. Incubate at room temperature for 20 minutes.
 - h. Apply vacuum (0.3 bar, in IST VacMaster®-96, Biotage, Uppsala, Sweden); extracts are collected (96-well Masterblock®, Greiner Bio One, Frickenhausen, Germany). Extracts are then transferred to labelled tubes.
 - i. Eluates are dried down under nitrogen at 37°C (SPE Dry™ 96 Dual, Biotage, Uppsala, Sweden). **Samples can be frozen at this point for later analysis.**
- vi. Derivatisation
- a. Add 200 μ L pyridine:acetic anhydride (1:1, v/v as above) to the dried extracts.
 - b. Incubate at room temperature for 15 minutes.
 - c. Dry down under nitrogen at 37°C as above.
 - d. Reconstitute in 100 μ L of 5% acetic anhydride in heptane.

- vii. Transfer reconstituted samples to GCMS vials (200µL conical glass inserts, National Scientific, USA) and seal (11mm silver crimp cap, Agilent, UK).

The GC-MS system consisted of a Finnigan GC8000^{TOP} GC, an AS800 autosampler and a single quadrupole Voyager mass spectrometer operated via Xcalibur (Version 1.2). The analytical column was an HP-Innowax column (30m x 0.32mm x 0.25µm; Agilent, Berkshire, UK). The sample was injected using splitless injection in a 1µL volume. Injection temperature was 260°C. The column oven temperature was 60°C at injection, programmed to reach 150°C at a rate of 30°C per minute then further programmed to reach 260°C at a rate of 10°C per minute with a final hold time of 3 minutes. Source temperature was 175°C. Helium gas was used as the mobile phase at a rate of 2.5mL per min.

Mass spectra were acquired using negative chemical ionisation with methane (BOC, Glasgow, UK) as the reagent gas. Selective ion monitoring was used with a total run-time of 18 minutes. Glycerol, d5-glycerol and butanetriol were monitored from 0 – 10 minutes; glucose, d2-glucose and ¹³C₆-glucose were monitored from 10-18 minutes. The following ions were monitored: derivatised glucose (*m/z* 287), d2-glucose (*m/z* 289), glycerol (*m/z* 217), d5-glycerol (*m/z* 222) and internal standards ¹³C₆ glucose (*m/z* 293) and butanetriol (*m/z* 231). Glucose, d2-glucose and ¹³C₆-glucose were detected as isomers with retention times of 15.01 and 15.33 minutes.

2.7.AMPOP study: Magnetic Resonance Imaging Techniques

Magnetic Resonance Imaging (MRI) is a technique which uses the properties of atomic nuclei within magnetic field to generate images. It has advantages over other imaging modalities such as X-ray, computed tomography and ultrasound in that it produces higher resolution images of soft tissues and does not require the use of ionising radiation. This last feature makes it particularly useful for studies in pregnant women. The ability to resolve soft tissues of different types makes this technique useful for identification of adipose tissue and it was used in the studies reported in this thesis to quantify intra-abdominal adipose.

Further detail regarding the specific chemical composition of regions of interest can be gained from the use of complementary techniques such as magnetic resonance spectroscopy (MRS). This technique makes use of the properties of specific isotopes within different chemical species. In this study, we used ^1H -MRS, but other magnetic isotopes can also be used, such as ^{13}C , ^{31}P and ^{19}F . The behaviour of each ^1H is influenced by the chemical structure of the molecule which it is part of; this allows characteristic spectra to be generated which correspond to specific metabolites of interest; in this study, triglyceride and water. Not only does this technique allow identification of specific metabolites, but also their quantification; thus we are able to quantify intra-organ lipid content by this method.

In this study, we used a 3 Tesla (3T) magnet. This has a stronger magnetic field than most MRI systems which are used for routine clinical purposes (commonly 1.5T systems). With respect to patient safety for scanning at 3T, the following were considerations. Firstly, a degree of heating of the subject

occurs during MR imaging; the effect of this is minimised by keeping the scanning room air conditioned, minimising scan time and keeping scan subjects loosely clothed while being scanned. In pregnant patients, the effect of excess heat on fetal development in the first trimester is not known, and imaging of pregnant patients at 3T is not currently recommended. In the third trimester, the effect of heating on the fetus was not thought to be a significant problem provided that steps were taken to minimise heat accumulation. Theoretical estimates of heat accumulation in fetal tissues secondary to radiofrequency exposure suggest that temperature rises would be lower than expected to cause teratogenesis, however, these estimates rely on models derived from sheep studies and therefore there is uncertainty regarding associated risk (Gowland and De Wilde, 2008). Mathematical modelling of the heat exchange in a human pregnant model suggests that as long as acceptable maternal exposure limits are adhered to, fetal exposure would also remain within those acceptable limits (Hand, Li, *et al.* 2010). Secondly, the MR system is noisy and aural protection was provided for subjects within the scanner. The impact of excess noise on fetal hearing is thought to be minimal; no patients reported any specific fetal responses to noise during scan sessions. There are few studies of long term follow up of infants exposed to MRI antenatally; those which have been carried out have not shown any detrimental effects on neurological development or hearing but small sample size makes it difficult to definitively prove absence of harm (Clements, Duncan, *et al.* 2000; De Wilde, Rivers, *et al.* 2005).

2.7.1. Patient recruitment

Patients were recruited from the Metabolic Antenatal Clinic and control cohort as described above. Pregnant women were imaged at approximately 36 weeks gestation, (for demographic details, see Chapter 5). All had healthy singleton pregnancies, with a normal OGTT at 28 weeks and no contra-indications to MR scanning.

2.7.2. Study Protocol

Patients were imaged at the Clinical Research Imaging Centre at the Royal Infirmary of Edinburgh on a Siemens Magnetom Verio 3T Whole Body MRI scanner (Siemens Medical, Erlangen, Germany). Patients were positioned in the scanner in a left lateral position to prevent aorto-caval compression. MRI data was obtained using a combination of body matrix and spine matrix coil elements. The body matrix coil was positioned over the maternal liver and thigh. Aural protection was provided by use of ear plugs and headphones. Contact was maintained with the scanning staff at all times through the use of an intercom. Heart rate and SpO₂ were continuously monitored during the scan process; blood pressure was recorded prior to commencing scanning and every 10 minutes thereafter.

MRI and MRS was acquired a combination of body matrix and spine matrix elements. 2D multi-slice FLASH images were acquired axially central to the right lobe of the maternal liver with water and lipid signals in and out of phase, using echo times of 2.46, 4.92, and 8.61ms. MRS was also acquired in maternal right quadriceps.

2.7.3. Assessment of Intra-abdominal Adipose

To define subcutaneous and intra-abdominal fat, a 3D T1-weighted VIBE sequence was acquired axially through the liver with in and out phase images, and lipid signals defined using a combination of the in and out of phase images with a semi-automated thresholding technique employed using the commercial software SliceOmatic™ (TomoVision, Quebec, Canada).

Adipose tissue appears bright on T1-weighted images. Regions of interest with signal intensity above an investigator-defined threshold were coloured to delineate intra-abdominal adipose (red) and subcutaneous adipose (green) and paraspinal adipose (blue) (Figure 2.1). The volume of the coloured areas can be calculated and expressed as a percentage of the total abdominal volume. Intra-abdominal adipose is defined as all adipose within the abdominal cavity, and does not distinguish between intra- and retro-peritoneal deposits. Subcutaneous adipose is defined as all adipose inferior to skin but superior to abdominal cavity. Where breast tissue is in the field of view, this was excluded from analysis. Intra-observer variability was assessed by the same observer defining the same image on 10 separate occasions inter-observer variability was assessed by comparison of data of 2 independent observers of the same subject (see Chapter 5.3.3).

For the studies in this thesis, there are several factors influencing our approach. This is discussed in further detail in Chapter Five. In brief: firstly, patients are positioned in a left-lateral tilt rather than supine. Secondly, at 36 weeks gestation, the gravid uterus takes up a large proportion of intra-

abdominal volume. Both of these contribute to a significant distortion of the normal female intra-abdominal anatomy, with both compression and elevation of organs in a variable distribution. This leads to issues with defining a region of interest. Firstly, the routine landmarks used for the non-pregnant population, while fixed, lie in the same region as the uterus. This limits their usefulness, as the majority of the field of view at this level will be composed of uterus and uterine contents rather than intra-abdominal organs, ie adipose. Additionally, acquisition of MR data is affected by motion artefact; contributions from fetal movement and amniotic fluid thus limit the usefulness of data in this region. We therefore endeavoured to identify a fixed landmark cranial to this. Images were reviewed by 2 investigators (Sarah Barr and Calum Gray) and by a consultant obstetric radiologist (Jane Walker). The left renal pelvis was chosen as a suitable landmark (a) as it was clearly identifiable in all patients, (b) since it is a retro-peritoneal organ, it is less affected by the presence of a gravid uterus and (c) it is sufficiently cranial to uterine fundus that acceptable data quality is maintained. The left renal pelvis is usually located at the position of the first lumbar vertebra.

Following identification of a fixed anatomical landmark, a multi-slice approach was used. It was felt that a single-slice approach would be sufficiently non-representative to outweigh the validity of any data and that a multi-slice approach which included the uterus would be sufficiently affected by artefact that the additional data would not be reliable. Therefore a region composed of 18-20 x 3mm consecutive slices cranial to left renal pelvis were analysed using SliceOmatic™ as described as this avoided involving a region of the abdomen including the uterus.

Figure 2.1 Representative SliceOmatic™ images

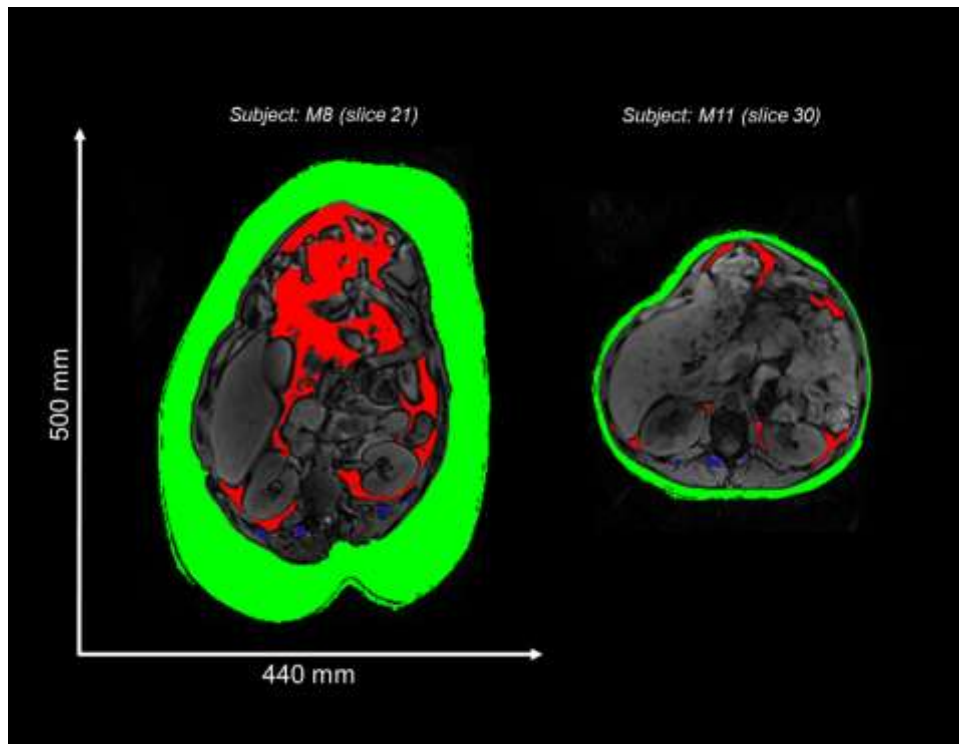


Figure 2.1 Representative abdominal slices from an obese (left) and a lean (right) subject. Green, subcutaneous adipose; red, intra-abdominal adipose; blue, para-spinal adipose.

2.7.4. Assessment of Intra-organ Lipid Content

2.7.4.1. ¹H-MR Spectroscopy

¹H proton MRS PRESS single voxel spectra were obtained in the right lobe of the liver and right quadriceps muscle remote from any large vessels using an echo time of 30 ms and a voxel size of 2cm³(liver) and 3cm³ (muscle). Spectra were obtained with and without water suppression, and lipid concentration was calculated from the water-suppressed acquisition using the spectroscopy analysis tool jMRUI.

2.7.4.2. In- and out-of-phase imaging

Intrahepatic lipid signal contribution was also calculated through subtraction of in and out of phase images (2.46ms and 8.61ms), and T2* decay during this time corrected using the two in-phase images (2.46ms and 4.92ms) according to an established protocol. This was carried out in addition to attempting to acquire ¹H-MRS data as there were difficulties in obtaining satisfactory spectra from all subjects. Due to the poor quality of hepatic spectra obtained, hepatic fat fraction data was derived using this method alone.

These calculations (from ¹H-MRS spectra and in- and out-of phase imaging) were performed by Dr Calum Gray; statistical analysis of the calculated lipid content data was performed by the investigator (SB).

Chapter Three

Adipose tissue function & metabolic adaptation in lean and obese pregnancy

3.1. Introduction

Pregnancy is associated with significant adaptation of maternal metabolism. In normal pregnancy, resistance to the action of insulin develops with increasing gestation, and is maximal in the third trimester (Catalano, Huston, *et al.* 1999). This development of insulin resistance (IR) is associated with changes in the function of adipose tissue, with increasing rates of lipolysis as this pathway is freed from inhibition by insulin. Enhanced lipolysis in the third trimester is important for normal fetal growth; reduced rates of lipolysis have been associated with fetal growth restriction and low birth weight (Diderholm, Stridsberg *et al.* 2005; Diderholm, Stridsberg *et al.* 2006).

Obesity is also associated with significant changes in lipid metabolism, and features increased systemic and intra-adipose inflammation, as well as alterations in circulating adipokines such as leptin and adiponectin. These systems drive local and systemic insulin resistance in obese subjects (Xu, Barnes *et al.* 2006; Antuna-Puente, Feve *et al.* 2008).

Whilst the drivers of maternal metabolic adaptation in pregnancy are not fully understood, there is modulation of normal metabolic regulatory systems by placental products, including those exclusive to the placenta such as placental lactogen, as well as those which are synthesized by both the placenta and by maternal tissues. For example, the placenta and fetal

membranes are a source of both leptin (Masuzaki, Ogawa, *et al.* 1997) and adiponectin (Caminos, Nogueiras *et al.* 2005), both of which are also synthesized by maternal adipose tissue. Inflammatory pathways may also be involved, directly or indirectly: the placenta is a significant source of several cytokines (Denison, Kelly, *et al.* 1998).

The aim of this study was to characterise the longitudinal changes in circulating metabolic and inflammatory markers in obese pregnant women at three different time points during pregnancy, and to evaluate the possible contribution of adipose tissue to circulating levels of these factors in the third trimester.

Where obesity and pregnancy co-exist, we hypothesised that:

1. Obese pregnant women have exaggerated insulin resistance in the third trimester compared with lean pregnant women
2. This is accompanied by exaggerated circulating adipokines and inflammatory markers.
3. Increased adipose tissue expression of adipokines and inflammatory markers in obese patients accounts for any observed increase in circulating levels of these factors.

To address these hypotheses, two parallel studies were undertaken. Firstly, we undertook a case-control study of obese and lean pregnant women. We recruited healthy pregnant women with a booking BMI > 40kg/m² and weight-matched controls with BMI between 20 and 25 kg/m². Women were seen at 3 time points during pregnancy, at 16, 28 and 36 weeks gestation. Anthropometric measurements were made and fasting blood samples

obtained. Delivery outcome and birth weight were recorded. All women had a normal OGTT at 28 weeks gestation (Section 2.1.3). Circulating adipokines, pro-inflammatory cytokine, glucose, insulin and NEFA were measured. Indices of insulin sensitivity were calculated.

Secondly, we carried out a cohort study of adipose tissue from pregnant women at term. Paired subcutaneous and omental adipose tissue biopsies were obtained from healthy, non-diabetic women undergoing an elective caesarean section delivery at term (> 37 completed weeks gestation). Transcript levels of adipokines, pro-inflammatory cytokines and genes involved in the regulation of lipid synthesis and storage were measured using real time RT-PCR and correlated with booking BMI.

3.2. Methods

3.2.1. Specimen collection

All biological samples were collected following informed consent from the donor. Ethical approval was obtained from the Lothian Research Ethics Committee (see Chapter 2.1.1).

3.2.1.1. Longitudinal samples

Fasting plasma and serum samples were collected as described in Section 2.2.1 from healthy women with singleton pregnancies enrolled in the Metabolic Antenatal Clinic and the accompanying control cohort. Women were excluded if they: had any pregnancy complications, such as gestational diabetes pre-eclampsia or obstetric cholestasis; had any endocrine comorbidities such as hypothyroidism or pre-existing diabetes; were on any regular medications with metabolic activities such as glucocorticoids, including oral and inhaled, or anti-inflammatory medications; if there were any concerns regarding abnormal fetal growth; if they delivered pre-term (prior to 37 completed weeks gestation). Participants attended 3 times during pregnancy, at 16, 28 and 36 weeks gestation. At each visit measurements were made of waist, hip, upper thigh and upper arm circumference; skinfold thicknesses at biceps, triceps and subscapular skinfolds were measured using Harpenden callipers and body composition (fat mass and fat free mass) estimated using bioelectrical impedance (TBF-300A Body Composition Analyser, Tanita, IL, USA). While every attempt was made to only include subjects with all measures and matching blood

samples, the number of subjects included varies slightly for some measures and assays; this was due to a small number of patients who either did not attend for one of the visits or in whom the blood samples were unsuitable for inclusion in analysis. Baseline characteristics are shown for the whole cohort in Table 3.1. No patients gave a history of polycystic ovarian syndrome (PCOS). However, a history of menstrual irregularity was present in a slightly greater proportion of obese compared to lean subjects (10/26 vs 7/24). This was not statistically significant ($p = 0.59$, Fisher's exact test). The prevalence of PCOS is an important consideration as this is a population of women with known increased risk of insulin resistance.

3.2.1.2. Term tissue samples

Paired subcutaneous and omental adipose biopsies were obtained as described in Section 2.2.2 from healthy women with singleton pregnancies of a range of booking BMI who were undergoing elective caesarean section delivery at term (37-42 completed weeks gestation). Tissue samples were immediately frozen at -80°C until use. Tissue samples were collected as part of a larger tissue collection protocol involving collection of a number of different tissue types for later inclusion in the Edinburgh Reproductive Tissue BioBank.

3.2.2. Plasma and serum analyses

A number of variables were analysed. These were specifically chosen either as markers of metabolic indices or inflammation for the larger source cohort of women attending the Metabolic Antenatal Clinic and the lean control cohort, or to answer specific questions only in the smaller nested cohort presented in this chapter. While the possibility of correlation by chance exists due to the number of variables being analysed, it was hoped that this would be minimised as each variable was selected to answer a specific question.

In the source cohort, C-reactive protein was measured as a non-specific marker of inflammation as a means of assessing the inflammatory environment in a larger population where specific cytokines were not being measured. Serum insulin, glucose and non-esterified fatty acids (NEFA) were also measured in the larger MAC cohort, as was alanine transferase (ALT), as an index of potential hepatic dysfunction within the population.

The specific assays performed in the nested cohort in this Chapter are detailed as follows.

3.2.2.1. ELISA assays

All ELISAs were carried out according to the general protocol specified in Chapter 2.5. Plasma samples using EDTA as anti-coagulant were used. All ELISAs were carried out according to the manufacturers instructions; kit manufacturer details are listed in Chapter 2.3.4. Pro-inflammatory cytokines interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and tumour necrosis factor alpha (TNF α) were chosen as these are commonly

associated with obesity and insulin resistance in both pregnant and non-pregnant populations.

Leptin and adiponectin were chosen as these are two key adipokines with associations with obesity and in the case of adiponectin, with the development of the insulin resistance of pregnancy. High molecular weight adiponectin was also analysed as there is some evidence to suggest that the relative proportion of high molecular weight to total adiponectin is more important in terms of its insulin sensitising activity (Fisher, Trujillo, *et al.* 2005).

3.2.2.1.1. *Interleukin 6 ELISA*

IL-6 was measured using high-sensitivity Quantikine® Colorimetric Sandwich ELISAs from R&D systems. The assay range was 10 to 0.156pg/mL; limit of detection was 0.04pg/mL; mean intra-assay CV was 6.5%, mean inter-assay was CV 10%.

3.2.2.1.2. *TNF α ELISA*

TNF α was measured using high-sensitivity Quantikine® Colorimetric Sandwich ELISAs from R&D systems. The assay range was 32 to 0.5 pg/mL; limit of detection was 0.1pg/mL; mean intra-assay CV 10.7%; mean inter-assay CV 22.5%.

3.2.2.1.3. *Monocyte Chemoattractant Protein 1 ELISA*

MCP-1 was measured using a Human MCP-1 ELISA kit from RayBio®. The assay range was 1000 – 31.2pg/mL; limit of detection was 2pg/mL; mean intra-assay CV was 6.2%; mean inter-assay CV was 1%.

3.2.2.1.4. *Leptin ELISA*

Leptin was measured using standard Quantikine® Colorimetric Sandwich ELISAs from R&D systems. All samples were diluted 1:100 prior to assay. The assay range was 1000 to 15.6pg/mL; limit of detection was 7.8pg/mL; mean intra-assay CV 11.2%; mean inter-assay CV 13.4%.

3.2.2.1.5. *Total Adiponectin ELISA*

In the circulation, adiponectin occurs in several forms. Upon secretion it self associates into trimers; the trimers can also associate to form hexamers and dodecamers. It is thought that the different species of adiponectin activate different signalling pathways and the high-molecular weight forms may have a role in vascular dysfunction in obesity (Kobayashi, Ouchi *et al.* 2004). Total adiponectin was measured using standard Quantikine® Colorimetric Sandwich ELISAs from R&D systems. This does not distinguish between the trimeric, hexameric or dodecameric forms of adiponectin. Samples from lean patients were diluted 1:400 prior to assay; samples from obese patients were diluted 1:100; control samples were diluted 1:200. The assay range was 250 to 3.9ng/mL; limit of detection was 0.25ng/mL; mean intra-assay CV was 9.4%; mean inter-assay CV 4.5%.

3.2.2.1.6. *High Molecular Weight Adiponectin ELISA*

High-molecular weight adiponectin was measured using a Human High Molecular Weight Adiponectin ELISA kit from Millipore. This ELISA requires pre-treatment of plasma samples with a specific protease enzyme to remove the hexameric and trimeric forms of adiponectin. This was carried out by incubating 20µL of plasma with 170µL of supplied digestion buffer and 10µL enzyme at 37°C for 2 hours. Digested samples are further diluted

1:10 with dilution buffer prior to use, and are thus assayed at 1:200 dilution. The assay range was from 200 to 1.56ng/mL; the limit of detection was 0.5ng/mL; mean intra-assay CV was 3.4%.

3.2.2.2. Plasma glucose, C-reactive protein and liver function analyses

Plasma glucose, C-reactive protein and liver function assays were carried out by the Biochemistry Laboratory of the Royal Infirmary of Edinburgh using an Abbott CD1600 Bioanalyser. Plasma glucose was measured using a hexokinase assay using plasma samples with fluoride oxalate.

3.2.2.3. Serum insulin and serum Non-esterified Fatty Acids

Fasting serum insulin was assayed in duplicate by using a commercially available ELISA kit (Mercodia, Uppsala, Sweden). The mean intra-assay CV was 8%. Serum NEFAs were assayed using a commercially available kit (Wako, USA).

3.2.3. Calculations

Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as follows for each patient:

$$\text{HOMA-IR} = (\text{Fasting Glucose} \times \text{fasting Insulin}) / 22.5$$

Where glucose is in mmol/L and insulin is in mU/L

Quantitative Insulin Sensitivity Check Index (QUICKI) was also calculated as follows for each patient:

$$\text{QUICKI} = 1 / (\log_{10} \text{Fasting Glucose}) + (\log_{10} \text{Fasting Insulin})$$

Where glucose is in mg/dL and insulin is in mU/L

3.2.4. Gene expression assays

3.2.4.1. RNA extraction

RNA extraction was carried out as described in Chapter 2.4.1.

3.2.4.2. cDNA synthesis

Complimentary DNA (cDNA) synthesis was carried out as described in Chapter 2.4.2.

3.2.4.3. Real time RT-PCR

Real time RT-PCR (Taqman®) was carried out as described in Chapter 2.4.3. Transcript levels of genes of interest were normalised to transcript levels of 18S or Cyclophilin A. A standard curve was generated for each gene using serially dilutions of pooled cDNA from all samples.

3.2.5. Statistical Analyses

All statistical analyses were carried out using GraphPad Prism® software. Data are presented as mean \pm SEM unless specifically stated. Demographic data were compared using Students' t-test. ELISA data were analysed using 2-way ANOVA with repeated measures where appropriate. Between-group comparisons were carried out with Bonferroni post-hoc testing. Real time RT-PCR data are expressed in arbitrary units; Rank-Spearman correlation was used as data were not normally distributed. Proportional data was analysed using Fisher's exact test. Significance was set at $p < 0.05$.

Transcript data was correlated with booking BMI rather than current adiposity. It is possible that adipose tissue characteristics may be influenced by recent depot expansion of the magnitude seen in pregnancy and while a more current measure of weight would have been ideal, both as a measure of current and also to provided information regarding weight gain in pregnancy, it was not possible to obtain this on the day of tissue collection due to a number of practical issues of access to the patient in a very busy clinical area. Additionally, routine weighing of pregnant women in the third trimester is not current practice and it is not possible to abstract this data retrospectively from the clinical record. A small number of patients recruited had also been attending the Metabolic Antenatal Clinic and had measures of weight and body composition made at 36 weeks gestation but as this represented only 5 subjects out of a cohort of a cohort of 45, it was of limited value. A pragmatic decision to use booking BMI only was made by the research team collecting a number of tissue types from patients attending for elective caesarean section to facilitate the recruitment process for the larger team.

Statistically significant differences between lean and obese women were found for most parameters measured, indicating adequate power in a cohort of this size. While statistically significant differences were detected between lean and obese subjects with respect to IL6 and MCP-1, there were no significant differences in plasma TNF α concentrations, although there was a trend towards greater TNF α in obese women. A sample size of 137 subjects in each group would have been required to give 80% power to show that such a difference was significant at the 5% significance level. A cohort of this size would not have been practical at this stage.

3.3. Results

3.3.1. Weight gain and anthropometric measurements in pregnancy

Table 3.1 summarizes the demographic characteristics of the longitudinal cohort. There was no significant difference in age, parity, mode of delivery or birth weight between lean and obese women. However, obese women had significantly higher systolic and diastolic blood pressure at 16 weeks (121 ± 2.1 vs 106 ± 1.6 mmHg and 68 ± 0.6 vs 63 ± 1.0 mmHg respectively, both $p < 0.001$).

The patient characteristics of the term tissue cohort are shown in Table 3.2

In the longitudinal study, changes in total body weight and fat mass are shown in Table 3.3. Lean women gained more weight than obese women between 16 and 36 weeks (10.2 ± 0.8 vs 7.5 ± 1.0 kg, $p = 0.05$). This represents a significantly higher percentage change in body weight from 16 to 36 weeks (16.7 ± 1.4 vs $6.4 \pm 0.9\%$, $p < 0.0001$).

Estimates of fat mass were made using bioelectrical impedance. Lean women gained more absolute fat mass than obese women, also representing a significantly higher percentage gain from 16 to 36 weeks (5.5 ± 2.8 vs 2.8 ± 0.8 kg, $p = 0.02$; 29.8 ± 3.6 vs $5.0 \pm 1.6\%$, $p < 0.0001$, respectively).

Skinfold thicknesses at biceps, triceps and subscapular skinfolds are shown in Figure 3.1.

Mean skinfold thickness increased slightly at each site between 16 and 36 weeks in lean women (biceps, 13.9 ± 1.0 vs 17.8 ± 2.2 mm; triceps, 15.3 ± 0.8 vs 18.3 ± 1.8 mm; subscapular, 14.3 ± 1.4 vs 17.0 ± 2.1 mm) although this did not reach statistical significance at any site. In obese women, mean biceps skinfold decreased between 16 and 36 weeks (34.8 ± 2.8 vs 29.5 ± 2.7 mm, not significant); mean triceps skinfold also decreased between 16 and 36 weeks (40.3 ± 3.5 vs 31.3 ± 3.8 mm, $p < 0.05$) and mean subscapular skinfold increased slightly (42.4 ± 6.3 vs 44.7 ± 8.2 mm, not significant).

Table 3.1 Demographic Characteristics of the Longitudinal Cohort

	Lean (n=24)	Obese (n=26)	p
Age (years)	33.6 ± 0.9	32.3 ± 1.0	0.3
Mean Parity	1+0	1+0	
Baseline Gestation (weeks + days)	15+6	16+2	0.55
Baseline BMI (kg/m ²)	22.7 ± 0.3	43.1 ± 0.5	<0.0001
Baseline Fat Mass (kg)	18.1 ± 0.9	57.7 ± 1.7	<0.0001
Baseline SBP (mmHg)	106 ± 1.6	121 ± 2.1	<0.0001
Baseline DBP (mmHg)	63 ± 1.0	68 ± 0.6	<0.0001
Gestation at Visit 2	28+4	28+2	0.23
Gestation at Visit 3	36+3	36+2	0.73
Gestation at delivery (weeks + days)	40+5	40+3	0.6
Birth weight (g)	3567 ± 120	3615 ± 113	0.8
Mode of delivery (n)			
SVD	13	11	0.55
Instrumental delivery	5	5	
Caesarean section (total/emergency)	6/4	10/5	

Table 3.1 Demographic characteristics of the longitudinal cohort. Data are presented as mean ± SEM unless otherwise stated. Lean and obese are compared using Students' t-test; proportional data regarding mode of delivery was analysed by Fisher's exact test..

Table 3.2 Demographic Characteristics of Term Tissue Cohort

	n = 45	Mean ± SEM	Range
Age (years)		32.9 ± 0.7	23 - 43
Parity (median)		1+	n/a
0+ (n)	n = 8		
1+ (n)	n = 26		
2+ or above (n)	n = 11		
Booking BMI (kg/m ²)		29.9 ± 1.1	20.7 – 48.8
Gestation at delivery (median)		39+1	37+1 – 41+4
Birthweight (g)		3578.5 ± 64.9	2770 - 4470

Table 3.2 Demographic characteristics of term cohort.

Data are presented as mean ± SEM, (range) unless otherwise specified.

Table 3.3 Weight gain and fat mass accretion in pregnancy

		Lean (n=24)	Obese (n=26)	p^a
Weight (kg)				
	16 weeks	61.4 ± 1.1	116.5 ± 2.6	<0.001
	28 weeks	68.2 ± 1.4	121.1 ± 2.8	<0.001
	36 weeks	71.6 ± 1.5 ^b	124.1 ± 2.9 ^c	<0.001
Mean weight gain (kg) 16 – 36 weeks		10.2 ± 0.8	7.5 ± 1.0	0.05
Weight gain (%) 16 – 36 weeks		16.7 ± 1.4	6.4 ± 0.9	<0.0001
Fat Mass (kg)*				
	16 weeks	18.1 ± 0.9	56.1 ± 1.7	<0.001
	28 weeks	26.0 ± 2.1	58.1 ± 1.9	<0.001
	36 weeks	23.5 ± 1.6 ^d	58.7 ± 2.1 ^e	<0.001
Mean gain in fat mass (kg) 16-36 weeks		5.5 ± 2.8	2.8 ± 0.8	0.02
Gain in fat mass (%) 16 – 36 weeks		29.2 ± 3.6	5.0 ± 1.6	<0.0001

Table 3.3 weight gain and fat mass accretion

a Lean compared to obese

b p <0.01 16 weeks compared with 36 weeks

c p <0.05 16 weeks compared with 36 weeks

d p < 0.01 16 weeks compared with 36 weeks (n = 17)

e not significant 16 weeks compared with 36 weeks (n = 17)

* Fat mass estimates were available at all 3 time points for n = 17 in each group; analysed using 2-way ANOVA with repeated measures.

Figure 3.1 Skinfold measurements

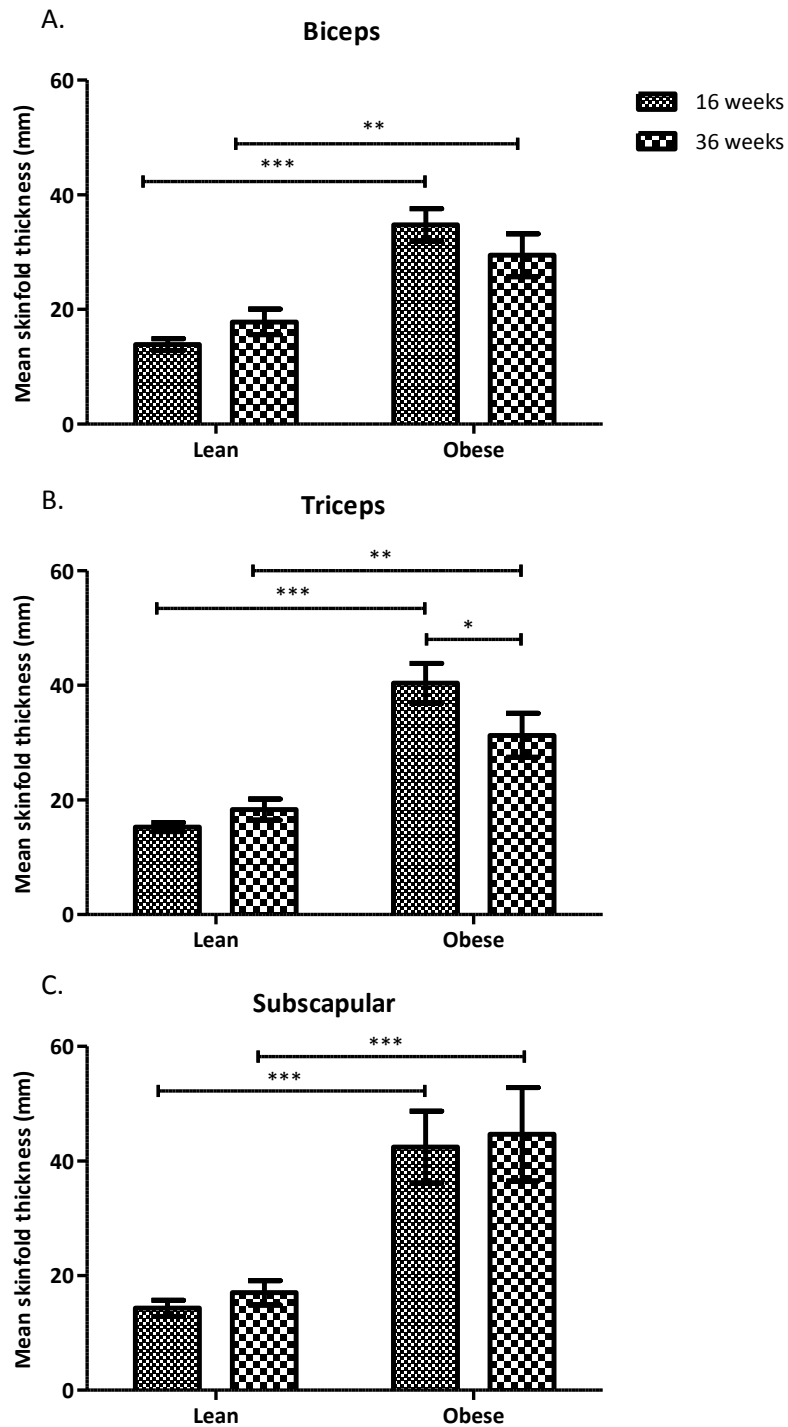


Figure 3.1 Longitudinal changes in skinfold thickness measurements.

A. Biceps skinfold; n = 19 (lean); n = 17 (obese); overall effect of BMI p < 0.0001

B. Triceps skinfold; n = 18 (lean); n = 17 (obese); overall effect of BMI p < 0.0001

C. Subscapular skinfold; n = 13 (lean); n=9 (obese) overall effect of BMI p < 0.0001

Data are presented as mean \pm SEM (mm); * p < 0.05 ** p < 0.01 *** p < 0.001

3.3.2. Longitudinal changes in fasting glucose, fasting insulin and indices of insulin sensitivity

Longitudinal changes in fasting glucose and insulin are shown in Table 3.4. All patients had a normal oral glucose tolerance test at 28 weeks gestation. There was no significant overall effect of BMI or gestation on fasting glucose. However, fasting plasma glucose was significantly greater at 36 weeks in obese compared with lean women ($p < 0.05$).

Fasting insulin was significantly greater in obese compared with lean women at all gestations ($p < 0.0001$). Both BMI and gestation had a highly statistically significant overall effect ($p < 0.0001$) on insulin. In lean and obese women, there was a significant increase in fasting insulin between 16 and 36 weeks ($p < 0.01$ and $p < 0.001$ respectively).

HOMA-IR and QUICKI scores were calculated (see Section 3.2.3) and are shown in Figure 3.2. HOMA-IR scores reflect insulin resistance; a higher score implies greater insulin resistance. QUICKI scores reflect insulin sensitivity; a higher score implies greater insulin sensitivity. HOMA-IR and QUICKI are derived from the same data, namely fasting glucose and fasting insulin and therefore express the same information but with an emphasis on either insulin resistance or sensitivity, respectively. Insulin resistance according to HOMA-IR scores was significantly greater in obese compared with lean women at all three gestations (Figure 3.2 A) ($p < 0.001$). There was a positive overall correlation of gestation with HOMA-IR ($p < 0.0001$); this was greater in obese women ($p < 0.001$) than lean women ($p < 0.05$).

Table 3.4 Longitudinal changes in fasting glucose and insulin

	Lean (n = 24)	Obese (n = 26)	p
Fasting glucose (mmol/L)			
16 weeks	4.2 ± 0.06	4.4 ± 0.05	ns
28 weeks	4.3 ± 0.09	4.4 ± 0.05	ns
36 weeks	4.1 ± 0.08	4.4 ± 0.1	< 0.05
Fasting insulin (mU/L)			
16 weeks	4.4 ± 0.6	14.7 ± 1.3	< 0.0001
28 weeks	6.7 ± 0.5	16.2 ± 1.5	< 0.0001
36 weeks	6.9 ± 0.6	17.9 ± 1.4	< 0.0001

Table 3.4 Longitudinal changes in fasting glucose and insulin.

Data are presented as mean ± SEM.

Figure 3.2 Longitudinal changes in indices of insulin sensitivity

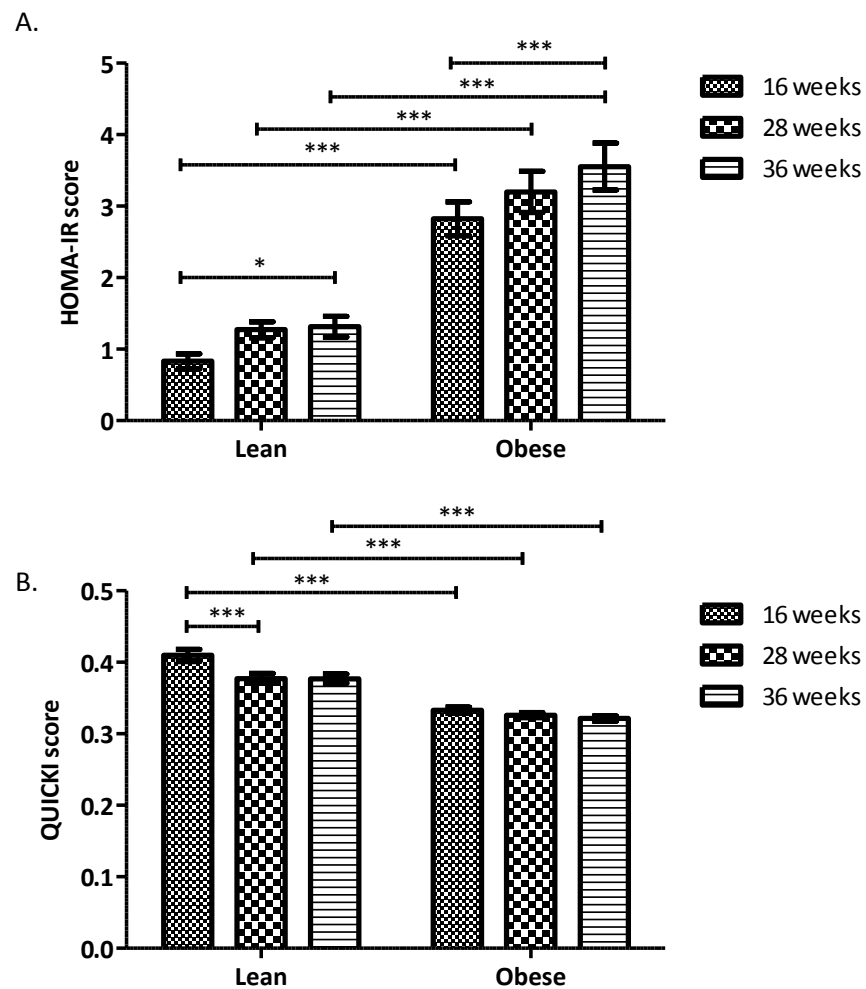


Figure 3.2 Longitudinal changes in indices of insulin sensitivity

A. HOMA-IR scores in lean and obese women at 16, 28 and 36 weeks.

B. QUICKI scores in lean and obese women at 16, 28 and 36 weeks.

Data are presented as mean \pm SEM. n = 24 (lean); n = 26 (obese).

* p < 0.05 *** p < 0.001 Data were analysed by 2-way ANOVA with repeated measures with Bonferroni post-hoc testing.

3.3.3. Longitudinal changes in leptin

Plasma leptin was greater in obese compared with lean women at all three gestations (Figure 3.3A) (at 16 weeks, 54.0 ± 8.9 vs 17.3 ± 2.4 ng/mL; at 28 weeks, 65.1 ± 7.6 vs 25.9 ± 4.6 ng/mL; at 36 weeks, 72.7 ± 8.5 vs 24.7 ± 4.5 ng/mL, all $p < 0.001$). There was a significant increase in plasma leptin in obese patients between 16 and 36 weeks (54.0 ± 8.9 vs 72.7 ± 8.5 ng/mL, $p < 0.05$); but no such increase with gestation in lean women.

Transcript levels of leptin were not significantly correlated with BMI in either subcutaneous or omental adipose tissue at term (Figure 3.3 B&C).

3.3.4. Longitudinal changes in adiponectin

Total plasma adiponectin was significantly greater in lean compared with obese women at 16 weeks (Figure 3.4A) (9.4 ± 1.0 vs 5.8 ± 0.60 μ g/mL, $p < 0.001$); however, at 28 and 36 weeks there was no significant difference between lean and obese women (6.1 ± 0.51 vs 5.5 ± 0.51 μ g/mL at 28 weeks; 6.3 ± 0.70 vs 5.1 ± 0.41 μ g/mL at 36 weeks). There was a significant reduction in total plasma adiponectin from 16 to 28 weeks in lean women (9.4 ± 1.0 vs 6.1 ± 0.51 μ g/mL, $p < 0.001$); however, there was no such reduction in obese women.

At term, there was no significant correlation between transcript levels of adiponectin and BMI in subcutaneous or omental adipose tissue (Figure 3.4 B&C).

High molecular weight adiponectin was measured in the same samples as total adiponectin and the ratio of the two calculated (Figure 3.5). Similar to the case with total adiponectin, there was a significant decrease in high molecular weight adiponectin in lean women between 16 and 28 weeks, but no further decrease (5.1 ± 0.5 , 2.3 ± 0.3 and 2.4 ± 0.4 $\mu\text{g/mL}$ at 16, 28 and 36 weeks respectively, $p < 0.001$ between 16 and 28 weeks). Obese women had slightly lower high-molecular weight adiponectin at 16 weeks compared with lean women, though this is not statistically significant, and no change with gestation (4.0 ± 0.8 , 3.25 ± 0.5 and 3.7 ± 0.5 $\mu\text{g/mL}$ at 16, 28 and 36 weeks respectively). Lean women had a significantly lower ratio of high molecular weight to total plasma adiponectin at all gestations (0.38 ± 0.01 vs 0.63 ± 0.02 ; $p < 0.001$). This did not change with gestation.

Figure 3.3 Longitudinal changes in leptin & third trimester adipose leptin mRNA

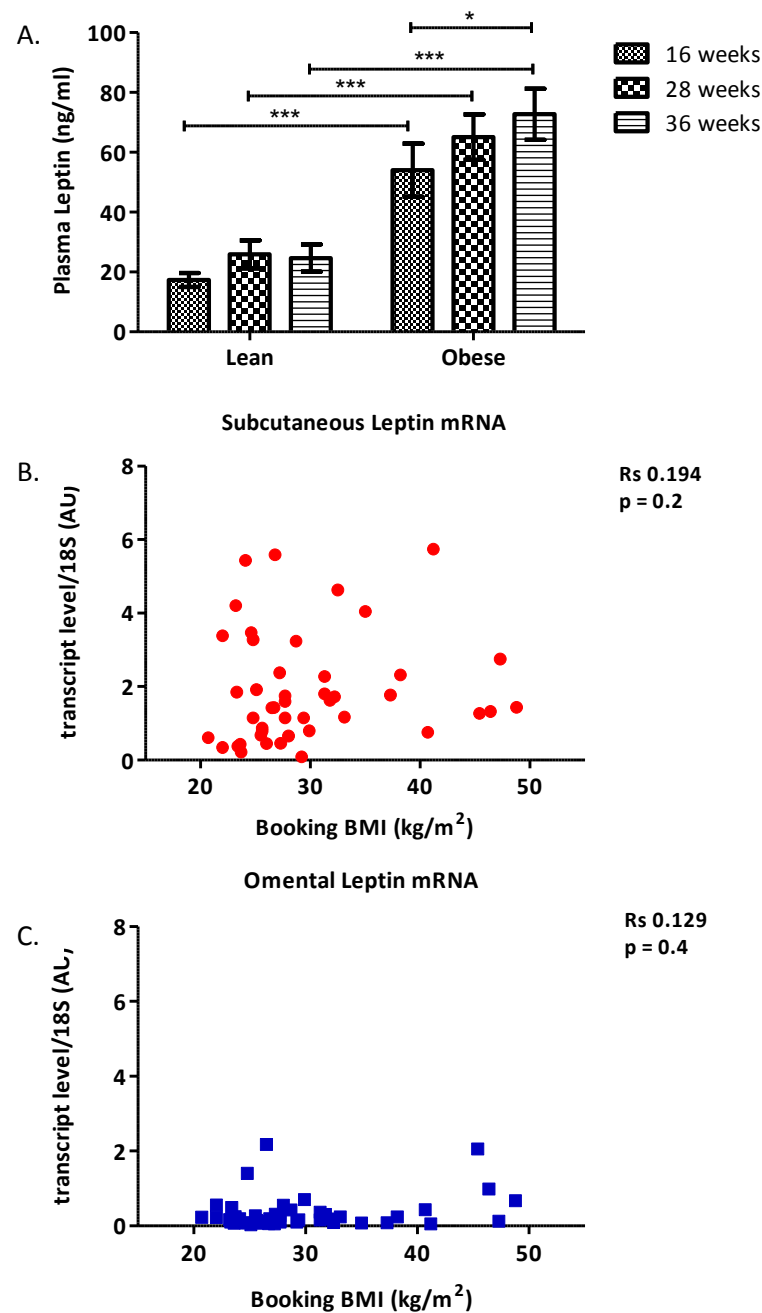


Figure 3.3 Longitudinal changes in circulating leptin and third trimester adipose leptin mRNA

A. Fasting plasma leptin. Data are presented as mean \pm SEM (ng/mL). $n = 14$ (lean); $n = 12$ (obese). * $P < 0.05$ ** $p < 0.001$ Data were analysed by 2-way ANOVA with repeated measures with Bonferroni post-hoc testing.

B & C. Transcript levels of leptin in paired subcutaneous (B) and omental (C) adipose tissue at term obtained during elective caesarean section delivery. Transcript levels are expressed in arbitrary units (AU), relative to transcript levels of 18S; $n=45$. Data were analysed by Rank-Spearman correlation.

Figure 3.4 Longitudinal changes in adiponectin & third trimester adipose adiponectin mRNA

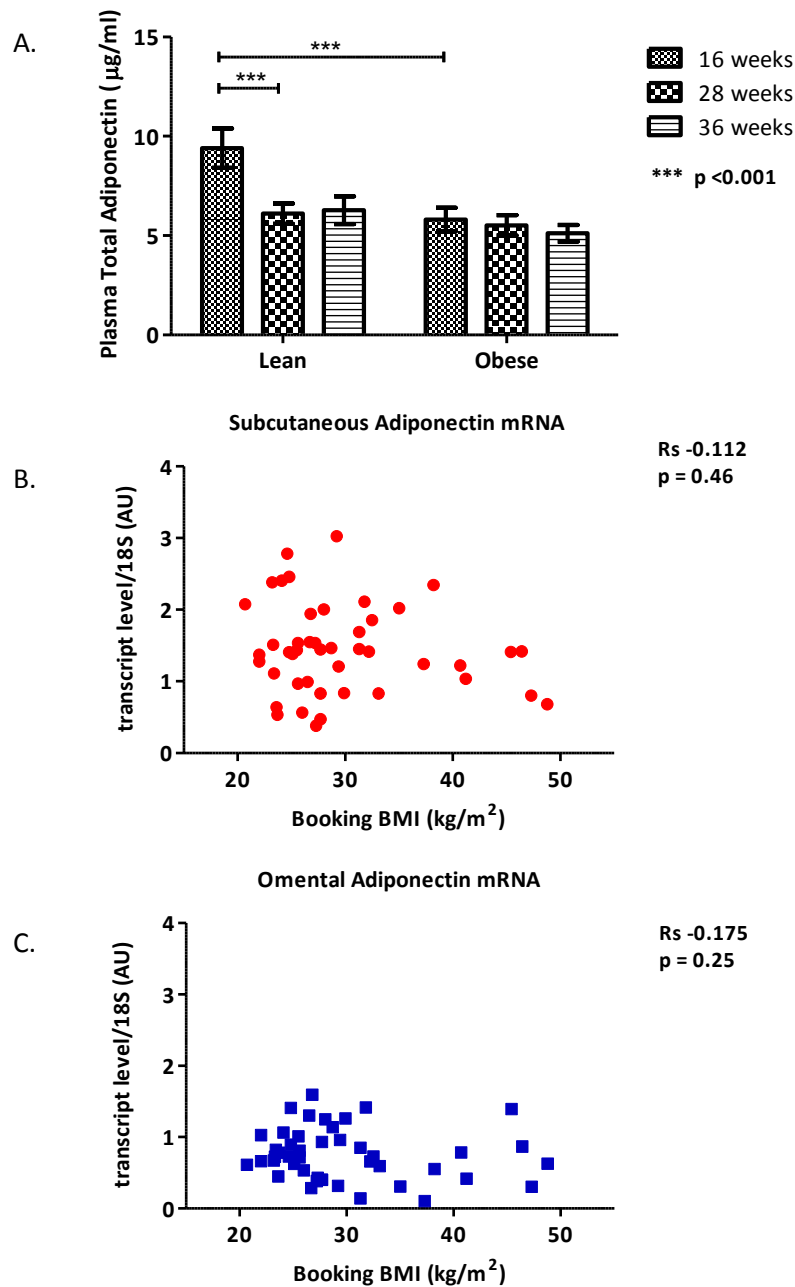


Figure 3.4 Longitudinal changes in circulating adiponectin and third trimester adipose adiponectin mRNA

A. Fasting total plasma adiponectin. Data are presented as mean \pm SEM ($\mu\text{g/ml}$), $n=18$ per group. Data were analysed by 2-way ANOVA with repeated measures with Bonferroni post-hoc testing.

B & C. Transcript levels of adiponectin in paired subcutaneous (B) and omental (C) adipose tissue biopsies at term; transcript levels are expressed in arbitrary units (AU), relative to transcript levels of 18S; $n=45$. Data were analysed by Rank-Spearman correlation.

Figure 3.5 Longitudinal changes in total and high molecular weight adiponectin

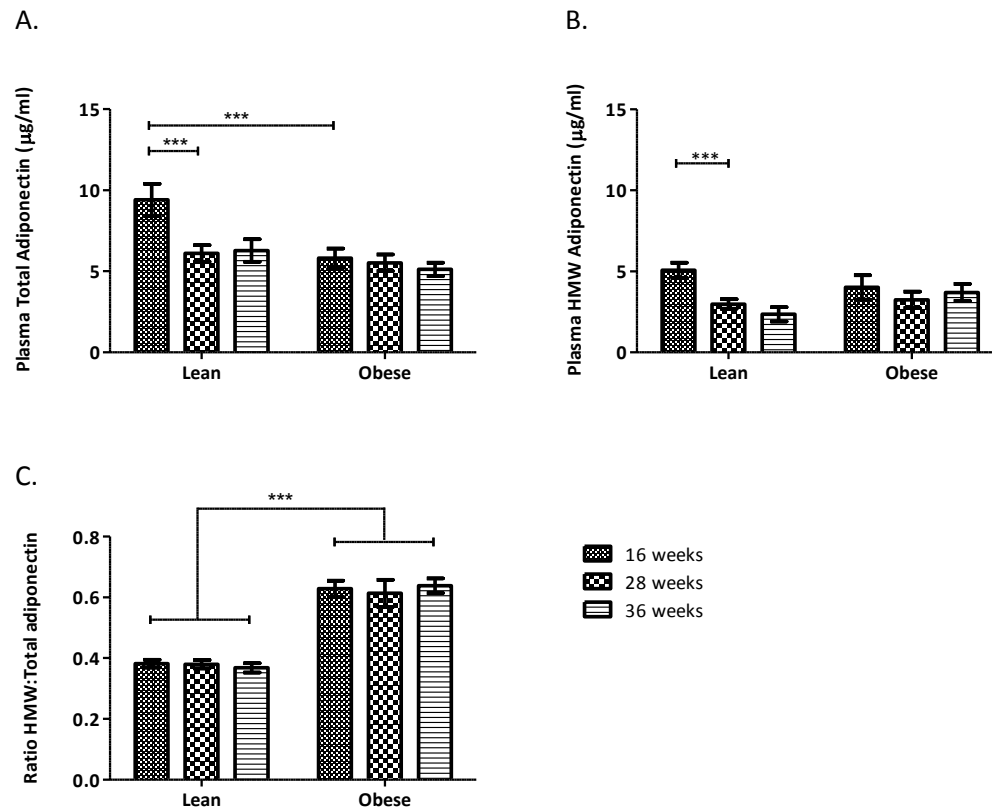


Figure 3.5 Longitudinal changes in circulating total and high molecular weight adiponectin

A. Fasting total plasma adiponectin.

Data are presented as mean \pm SEM ($\mu\text{g/ml}$), n=18 per group.

B. High Molecular weight adiponectin.

Data are presented as mean \pm SEM ($\mu\text{g/mL}$). n=6 per group. ***p<0.001

C. Ratio of high molecular weight:total plasma adiponectin.

Data are presented as mean \pm SEM; n=6 per group. *** p<0.001

Data were analysed by 2-way ANOVA with repeated measures with Bonferroni post-hoc testing.

3.3.5. Longitudinal changes in inflammatory markers in pregnancy

Plasma IL-6 increased with gestation in lean women (Figure 3.6 A) (0.84 ± 0.07 , 1.5 ± 0.2 and 1.7 ± 0.2 pg/mL at 16, 28 and 36 weeks respectively, $p < 0.01$ between 16 and 36 weeks). Plasma IL-6 also increased in obese women with gestation (1.8 ± 0.1 , 2.1 ± 0.2 and 3.3 ± 0.4 pg/mL at 16, 28 and 36 weeks respectively, $p < 0.001$ between 16 and 36 weeks). Plasma IL6 was significantly greater in obese compared with lean women at 16 and at 36 weeks ($p < 0.01$ and $p < 0.001$, respectively).

At term, transcript levels of IL-6 were significantly positively correlated with booking BMI in subcutaneous adipose tissue ($R_s 0.43$, $p 0.003$, Figure 3.6 B) but not in omental adipose ($R_s 0.16$, $p = 0.3$, Figure 3.6 C).

Plasma MCP-1 increased with gestation in lean women (Figure 3.7 A) (17.6 ± 2.5 , 48.0 ± 4.7 and 60.3 ± 5.0 pg/mL at 16, 28 and 36 weeks respectively; $p < 0.01$ between 16 and 28 weeks). Plasma MCP-1 also increased with gestation in obese women (21.2 ± 2.9 , 56.9 ± 5.5 and 81.8 ± 8.5 at 16, 28 and 36 weeks respectively; $p < 0.001$ between 16 and 28 and 28 and 36 weeks). Obese women had significantly greater plasma MCP-1 than lean women at 36 weeks only ($p < 0.05$).

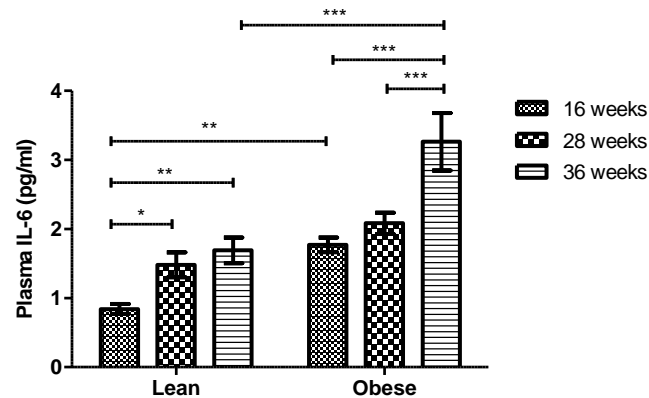
At term, transcript levels of MCP-1 were significantly positively correlated with booking BMI in subcutaneous adipose tissue ($R_s 0.33$, $p = 0.03$, Figure 3.7 B) but not in omental adipose ($R_s 0.09$, $p = 0.6$, Figure 3.7 C).

Plasma TNF α did not change with gestation in lean or obese women, and was not significantly different between lean or obese women at any gestation (Figure 3.8 A). At term, transcript levels of TNF α were not correlated with booking BMI in either subcutaneous or omental adipose tissue (Figure 3.8 B & C).

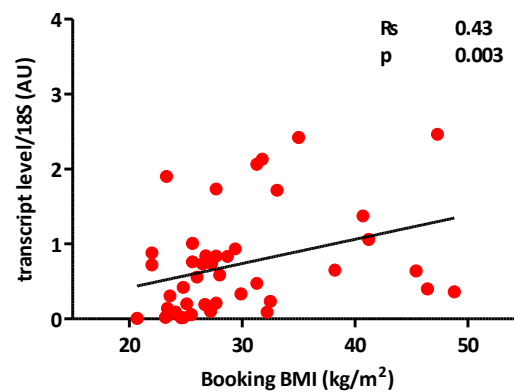
Serum CRP was significantly greater in obese compared with lean women at all three gestations ($p < 0.0001$), but there was no significant effect of gestation in either group (Figure 3.9).

Figure 3.6 Longitudinal changes in Interleukin-6 in pregnancy & third trimester adipose interleukin-6 mRNA

A.



B.



C.

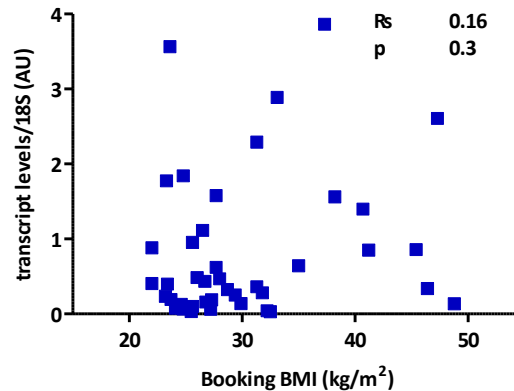


Figure 3.6 Longitudinal changes in circulating interleukin-6 and third trimester adipose interleukin-6 mRNA

A. Plasma IL-6 measured by ELISA. Data are presented as mean \pm SEM (pg/mL); n=19 (lean), n=20 (obese). * p<0.05 **p<0.01 ***p<0.001 Data were analysed by 2-way ANOVA with repeated measures with Bonferroni post-hoc testing.

B & C. Transcript levels of IL-6 in subcutaneous (B) and omental (C) paired adipose tissue biopsies obtained at elective caesarean section at term. Data are expressed in arbitrary units (AU) relative to transcript levels of 18S; n=45. Data were analysed by Rank-Spearman correlation.

Figure 3.7 Longitudinal changes in MCP-1 in pregnancy & third trimester adipose MCP-1 mRNA

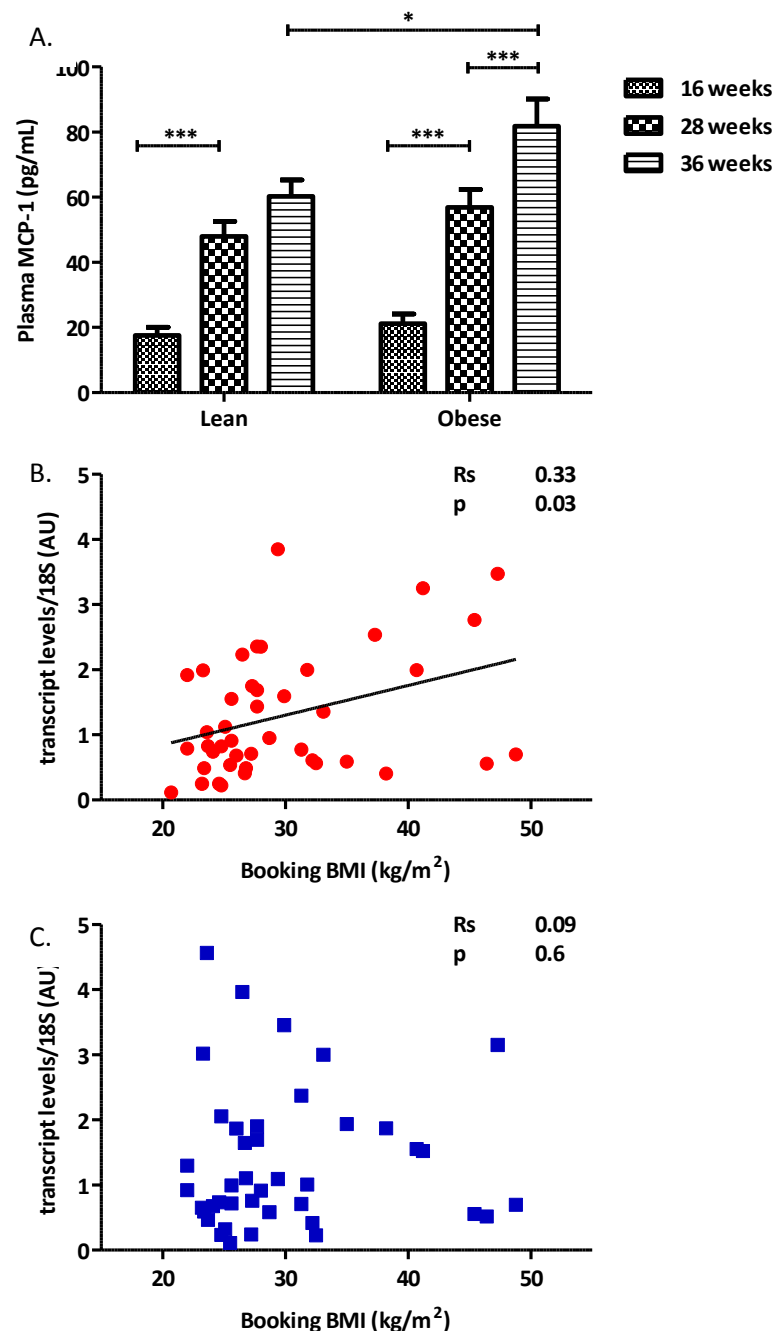


Figure 3.7 Longitudinal changes in circulating MCP-1 and third trimester adipose MCP-1 mRNA

A. Plasma MCP-1 measured by ELISA in lean and obese pregnant women. Data are presented as mean \pm SEM (pg/mL). $n = 16$ per group. * $p < 0.05$ *** $p < 0.001$ Data were analysed by 2-way ANOVA with repeated measures with Bonferroni post-hoc testing.

B & C. Transcript levels of MCP-1 in subcutaneous (B) and omental (C) paired adipose tissue biopsies obtained at elective caesarean section at term. Data are expressed in arbitrary units (AU) relative to transcript levels of 18S; $n = 45$. Data were analysed by Rank-Spearman correlation.

Figure 3.8 Longitudinal changes in TNF α in pregnancy & third trimester adipose TNF α mRNA

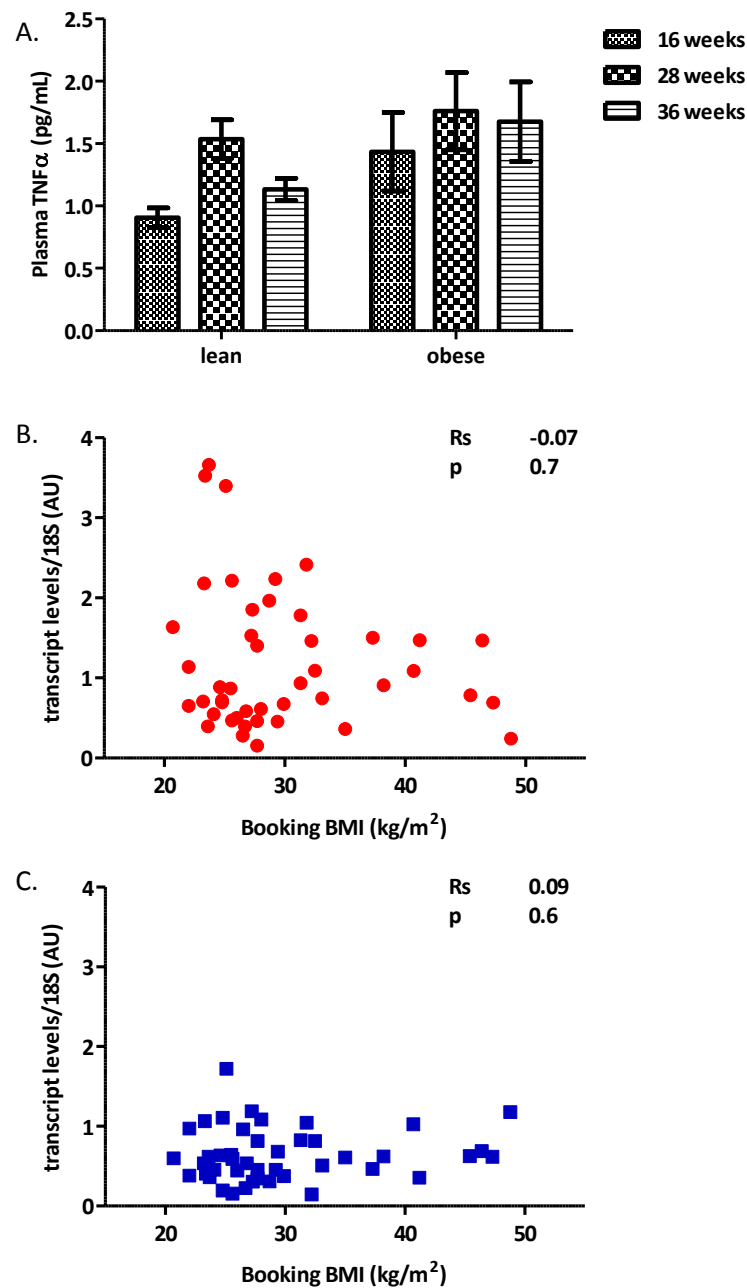


Figure 3.8 Longitudinal changes in circulating TNF α and third trimester adipose TNF α mRNA

A. Plasma TNF α measured by ELISA. Data are presented as mean \pm SEM (pg/mL). n=19 (lean), n=20 (obese). Data were analysed by 2-way ANOVA with repeated measures with Bonferroni post-hoc testing.

B & C. Transcript levels of TNF α in subcutaneous (B) and omental (C) paired adipose tissue biopsies obtained at elective caesarean section at term. Data are expressed in arbitrary units (AU) relative to transcript levels of 18S; n=45. Data were analysed by Rank-Spearman correlation.

Figure 3.9 Longitudinal changes in CRP in pregnancy

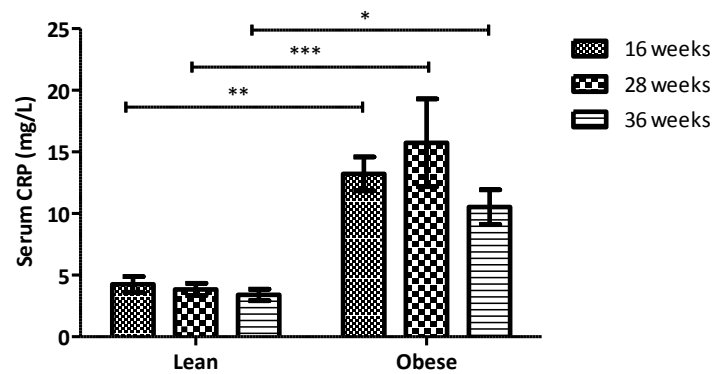


Figure 3.9 Longitudinal changes in CRP in pregnancy

Data are presented as mean \pm SEM (mg/L). n = 20 (lean); n = 23 (obese).

* p < 0.05 ** p < 0.01 *** p < 0.001

Data were analysed by 2-way ANOVA with repeated measures with Bonferroni post-hoc testing.

3.3.6. Longitudinal changes in Non-esterified Fatty Acids (NEFA) and ALT in pregnancy

Fasting serum NEFAs were greater in obese compared with lean women at all three gestations (Figure 3.10) (at 16 weeks, 0.29 ± 0.02 vs 0.53 ± 0.03 , $p < 0.001$; at 28 weeks, 0.27 ± 0.02 vs 0.45 ± 0.02 , $p < 0.001$; at 36 weeks, 0.36 ± 0.03 vs 0.49 ± 0.03 mmol/L, $p < 0.01$). There was a significant interaction between BMI and gestation ($p = 0.02$). In lean women, there was a significant increase in NEFA from 28 to 36 weeks ($p < 0.01$); in obese women, there was a small decrease in NEFA from 16 to 28 weeks ($p < 0.05$).

Serum ALT was measured at 16, 28 and 36 weeks (Figure 3.11). Obese women had significantly greater serum ALT at 16 weeks compared with lean women (23 ± 3.2 vs 15.8 ± 1.1 IU/L, $p < 0.05$); There was a significant reduction in serum ALT between 16 and 28 weeks in obese women (23.0 ± 3.2 , 18.5 ± 1.8 and 17.4 ± 1.6 IU/L at 16, 28 and 36 weeks, respectively, $p < 0.01$). There was no effect of gestation in lean women (15.8 ± 1.1 , 16.4 ± 1.4 and 15.4 ± 0.9 IU/L at 16, 28 and 36 weeks, respectively).

3.3.7. Fatty acid metabolism in adipose tissue at term

Transcript levels of genes coding for enzymes involved in fatty acid synthesis were quantified in subcutaneous and omental adipose tissue at term (Table 3.5). There was a statistically significant negative correlation between transcript levels of fatty acid synthase (FASN) and BMI in both subcutaneous ($R_s -0.33$, $p = 0.03$) and omental ($R_s -0.32$, $p = 0.03$) adipose tissue at term (Figure 3.12).

Figure 3.10 Longitudinal changes in NEFA in pregnancy

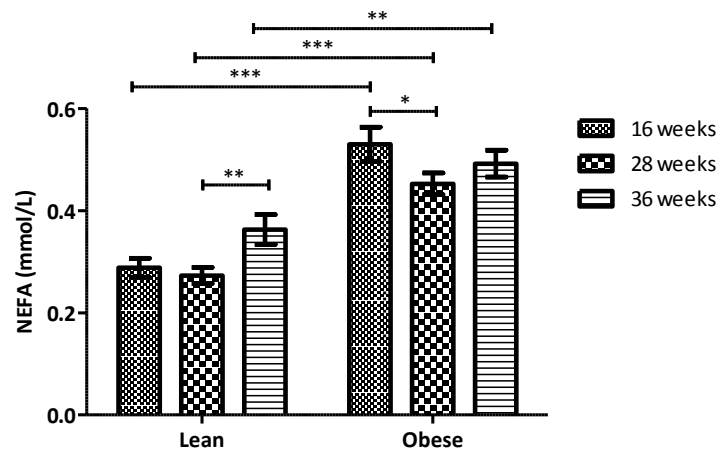


Figure 3.10 Longitudinal changes in NEFA in pregnancy

Data are presented as mean \pm SEM (mmol/L). n = 24 (lean); n = 25 (obese).

* p < 0.05 ** p < 0.01 *** p < 0.001

Data were analysed by 2-way ANOVA with repeated measures with Bonferroni post-hoc testing.

Figure 3.11 Longitudinal changes in ALT in pregnancy

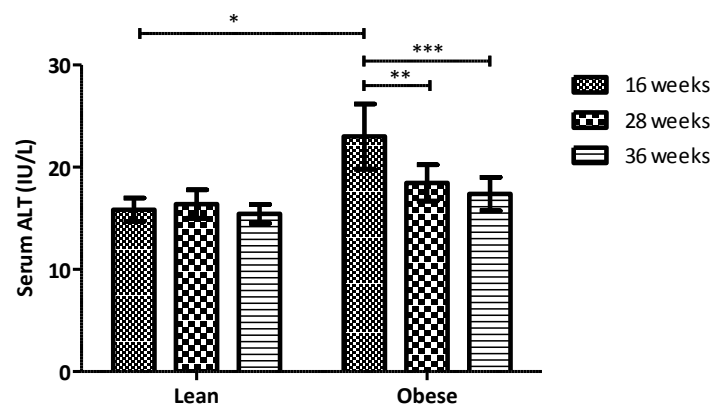


Figure 3.11 Longitudinal changes in alanine transferase in pregnancy.

Data are presented as mean \pm SEM (IU/L). n = 23 (lean); n = 24 (obese).

* p < 0.05 ** p < 0.01 *** p < 0.001

Data were analysed by 2-way ANOVA with repeated measures with Bonferroni post-hoc testing.

Figure 3.12 Transcripts of Fatty Acid Synthase (FAS) in adipose tissue at term

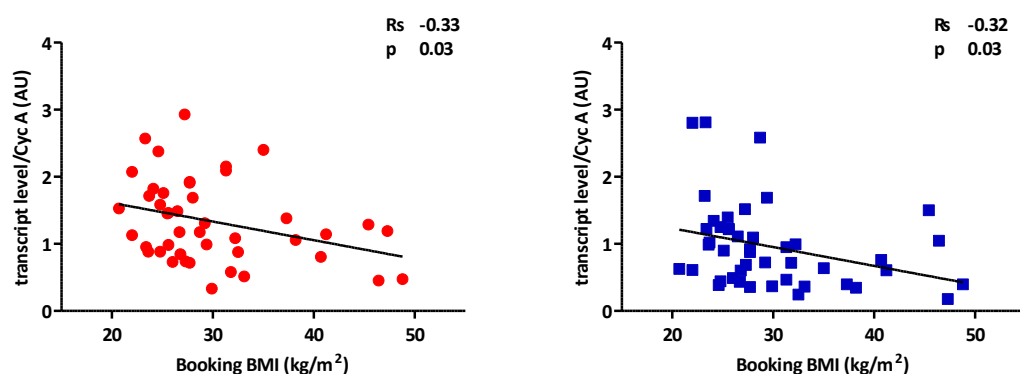


Figure 3.12 Transcript levels of FAS in adipose tissue at term.

A & B. Transcript levels of FAS in in subcutaneous (A) and omental (B) paired adipose tissue biopsies obtained at elective caesarean section at term.

Data are expressed in arbitrary units (AU) relative to transcript levels of cyclophilin A; n=45. Data were analysed by Rank-Spearman correlation.

Table 3.5 Transcript levels of genes regulating lipid storage, synthesis and local adipose tissue metabolism

Gene	Role	Subcutaneous		Omental	
		<i>Rs</i>	<i>p</i>	<i>Rs</i>	<i>p</i>
FAS	Fatty acid synthase (Figure 3.12)	-0.33	0.03	-0.32	0.03
RBP4	Levels positively correlate with fat mass and inversely correlate with insulin sensitivity (Yang, Graham <i>et al.</i> , 2005; Kotnik, Fischer-Posovszky <i>et al.</i> , 2011)	-0.10	0.5	0.04	0.8
PPAR alpha	Nuclear receptor; involved in regulation of fatty acid oxidation (Van Raalte, Li <i>et al.</i> 2004)	0.12	0.4	0.02	0.9
DGAT2	Catalyses rate-limiting step in triglyceride synthesis (Yen, Stone <i>et al.</i> 2008)	-0.07	0.6	-0.10	0.5
ME1	Generates NADPH for fatty acid synthesis (Wise & Ball, 1964)	-0.01	0.9	-0.16	0.3
SCD1	Required for unsaturated fatty acid synthesis; involved in response to leptin (Paton & Ntambi, 2009)	0.10	0.5	-0.04	0.8
11 β -HSD1	Enzyme catalysing the interconversion of cortisol to cortisone; involved in regulating the local availability of active steroid in adipose tissue (Seckl, Morton <i>et al.</i> 2004)	0.03	0.8	-0.02	0.9
GR	Nuclear receptor for glucocorticoids (Macfarlane, Forbes <i>et al.</i> 2008)	-0.04	0.8	-0.08	0.6
LPL	Involved in the hydrolysis of circulating lipoproteins (Wang & Eckel, 2009)	-0.19	0.2	-0.17	0.3
HSL	Rate limiting step in lipolytic cascade; catalyses hydrolysis of triglyceride to diglyceride (Kraemer & Shen, 2002)	0.15	0.3	-0.17	0.3
ATGL	Novel triglyceride lipase (Zechner, Kienesberger, <i>et al.</i> 2008)	0.27	0.07	0.10	0.5
CD 68	LDL receptor; Monocyte cell line marker; upregulated in obesity and insulin resistance (Di Gregorio, Yao-Borengasser <i>et al.</i> 2005)	-0.02	0.89	0.26	0.08

Transcript levels as determined by qRT-PCR were correlated with booking BMI using Rank Spearman correlation.

3.4. Discussion

In this chapter we have shown in response to pregnancy, there were significant changes to maternal metabolic characteristics. In lean women, adipose tissue underwent a large expansion with similar changes in adipokine profile as observed in non-pregnant individuals with excess adipose accumulation. By the third trimester, lean women had evidence of excess inflammation, hyperlipidaemia and insulin resistance. In keeping with our hypotheses, obese pregnant women had evidence of exaggerated insulin resistance, inflammation and altered adipokine profile. However, there were some features of obese pregnancies which were not in keeping with the hypothesis that altered adipose tissue function alone is sufficient to account for these differences.

3.4.1. Weight gain in pregnancy

Weight gain is a feature of normal pregnancy due to growth of the fetus, placenta and uterus, as well as expansion of fluid volumes including amniotic fluid and circulating blood volume. Weight gain in pregnancy is highly variable, with estimates of 6-18kg reported for normal weight women (Taggart, Holliday *et al.* 1967; Nutrition During Pregnancy, Institute of Medicine, 1990; Dawes & Grudzinskas, 1991, Bergmann, Flagg *et al.* 1997, Kopp-Hoolihan, van Loan *et al.* 1999, Soltani and Fraser, 2000; Eriksson, Löf *et al.* 2010). Relative contributions from different tissues are estimated at approximately 26% from fetus, 4% from placenta, 6% from amniotic fluid, 8% from uterus, 12% from expansion of blood volume 12% from retention of

extracellular water, with the remainder as fat mass (Hyttén & Chamberlain, 1991).

It has also been recognised that pre- or early pregnancy BMI is associated with differences in gestational weight gain (Bergmann, Flagg *et al.* 1997; Cedergren, 2006). In our cohort of women, subjects with a booking BMI $>40\text{kg/m}^2$ gained significantly less weight than normal weight women. There is also known to be a significant increase in maternal fat stores, particularly in the second trimester (Pipe, Smith *et al.* 1979, Kopp-Hoolihan, van Loan *et al.* 1999, Soltani & Fraser, 2000), with utilization of maternal fat in later pregnancy when fetal energy requirements increase. On average, women gain 2-5kg of fat mass (Hyttén & Chamberlain, 1991, Kopp-Hoolihan, van Loan *et al.* 1999, Ehrenberg, Huston-Presley *et al.* 2004). Similar to overall weight gain, we have shown that morbidly obese women gain less total fat mass than normal weight women. In normal weight women, this gain in fat mass represents a significant expansion of their pre-pregnancy depots, with an average 29% increase in fat mass from 16 to 36 weeks, compared with an average 5% increase in overall fat stores over the same time in morbidly obese women. The difference between mean total weight gain and mean gain in fat mass was approximately 4-5kg for both lean and obese pregnant women (Table 3.3, p107); the additional weight gain in lean women is largely represented by additional fat mass.

Ehrenberg *et al* have previously shown that women who have a higher pre-gravid fat mass have a similar gain in fat mass compared to lean women (Ehrenberg, Huston-Presley *et al.* 2004). In a comparison of obese women who had normal glucose tolerance compared with those with gestational

diabetes, Okereke *et al* observed a mean gain in fat mass of approximately 3-4kg in both groups (Okereke, Huston-Presley *et al.* 2004). Soltani and Fraser observed a smaller increase in fat mass in obese compared with lean women (3.7 vs 4.9 kg), though this was not significant. In our cohort, obese patients gained significantly less fat mass than lean women (2.8 vs 5.5 kg). Discrepancies between studies may in part be due to differences in methodology between studies (estimation of fat mass using water displacement (Catalano, Wong *et al.* 1995; Ehrenberg, Huston-Presley *et al.* 2004; Okereke, Huston-Presley *et al.* 2004;) versus estimation from anthropometric measurements (Soltani & Fraser, 2000); versus bioelectrical impedance measurements (this study)). Assessment of body composition during pregnancy is difficult to carry out. The gold standard method of assessment of body composition, DEXA, is unsuitable during pregnancy due to use of ionising radiation, and therefore it is also difficult to assess the validity of the available methods during pregnancy.

Our morbidly obese patients also attended a high-risk antenatal clinic and there received serial measurements of weight at each visit, as well as advice regarding weight gain during pregnancy and dietetic input. It is possible that this intervention contributed to a smaller weight gain than might have been expected. However, it may also be a reflection of the greater initial fat mass of the obese patients in our cohort and genuine physiological inability to store excess calories in adipose tissue. Triglyceride storage in adipose tissue is insulin sensitive, and is therefore subject to suppression in insulin resistance states such as late pregnancy.

3.4.2. Body fat distribution

Previous studies have suggested that in pregnancy, fat mass is accumulated in the subcutaneous depot (Taggart, Holliday *et al.* 1967; Soltani & Fraser, 2000); although other studies which have included an evaluation of the visceral fat depot have indicated that visceral fat accumulation is also important (Kinoshita & Itoh, 2006). The greater overall skinfold thicknesses observed in our obese cohort supports a significantly greater subcutaneous adipose depot in these women compared with lean; however, it is not possible to assess visceral fat accumulation without the use of imaging studies.

Regional distribution of body fat is important due to regional variations in adipose tissue metabolism. In particular, the distinction between upper and lower body obesity is important: increased upper body or central fat distribution has a stronger correlation with increased risk of metabolic complications of obesity than lower body fat distribution (Jensen, 2008). In pregnancy, fat distribution tends to favour central body sites: increase in skinfold thicknesses are greatest in suprailiac and upper thigh sites compared either upper arm or lower thigh (Ehrenberg, Huston-Presley *et al.* 2003). However, lean women predominantly gained subcutaneous tissue in peripheral sites ie upper arm whereas obese women gained more central adipose tissue (suprailiac and subscapular) (Ehrenberg, Huston-Presley *et al.* 2003).

Upper body distribution of fat in obese subjects in pregnancy is associated with relative hyperinsulinaemia and earlier onset of maximal glucose

response than in lean women or obese women with a predominantly lower body adipose distribution (Landon, Osei *et al.* 1994). Upper body sites only were assessed in our cohort of patients; while obese women had significantly greater upper body fat distribution compared with lean women, the only difference was that in obese women triceps skinfold thickness was significantly reduced. This may reflect preferential deposition of adipose tissue in alternative sites in lean women.

3.4.3. Indices of insulin sensitivity

Insulin sensitivity was assessed using two methods in this study, HOMA-IR and QUICKI. Both these techniques make use of fasting insulin and fasting glucose levels and reflect fasting insulin sensitivity. QUICKI scores indicate insulin sensitivity, the greater the score, the higher the IS. Conversely, HOMA-IR scores reflect insulin resistance, the greater the score the greater the IR. The hyperinsulinaemic euglycaemic clamp (HEC) is the gold standard method of assessing insulin sensitivity. Insulin sensitivity assessed using this method in lean women increases slightly in early pregnancy, and then reduces by approximately 40% (Catalano, Huston *et al.* 1999). We have shown that throughout pregnancy, insulin sensitivity is lower in obese compared with lean women using both HOMA-IR and QUICKI assessments (Figure 3.6). Both these indices correlate well against the HEC technique (Muniyappa, Lee *et al.* 2007). The advantage of these indices is that they can easily be derived from a single fasting sample and are useful in population studies as well as for repeated measures. However, they do not provide information on the dynamic response to insulin which can be derived from clamp data.

In this cohort, insulin resistance, regardless of which fasting index is used, is a consistent feature of late pregnancy and in severely obese pregnant women, this is significantly exaggerated compared with lean women. The timing of onset of severe IR in obese women, and the extent to which it is more severe than in lean women is less clear. Furthermore, it is also evident that these differences occur in women with a normal 28 weeks glucose tolerance test who would otherwise be considered healthy in pregnancy,

illustrating the wide range of insulin resistance that can be tolerated in the pregnant women prior to onset of overt pathology.

3.4.4. Determinants of insulin resistance

3.4.4.1. Leptin in obese pregnancies

During pregnancy, serum leptin levels increase compared with non-pregnant women (Hardie, Trayhurn *et al.* 1997; Highman, Friedman *et al.* 1998; Hendler, Blackwell *et al.* 2005; McLachlan, O'Neal *et al.* 2006) and correlate with levels of estradiol and hCG, particularly in the first trimester, as well as with insulin sensitivity (McLachlan, O'Neal *et al.* 2006). Leptin concentrations continue to increase with gestation, with a lower rate of increase or a decline in the third trimester (Henson & Castracane, 2006); similarly, Sattar *et al.* observed a significant increase in plasma leptin in pregnant compared with non-pregnant women, with an increase in plasma leptin until approximately 30 weeks gestation, followed by a decline (Sattar, Greer *et al.* 1998). In lean women, these changes in circulating leptin levels correlate with changes in fat mass, with an increase to approximately 30 weeks followed by a decline in maternal fat mass, but with an overall greater fat mass persisting at 36 weeks compared with non-pregnant women (Eriksson, Löf *et al.* 2010).

We observed a similar pattern in plasma leptin in lean women, with an increase between 16 and 28 weeks in parallel with peak fat mass, followed by plateauing of circulating leptin at 36 weeks. However, in obese women, we observed a different pattern of circulating leptin: plasma leptin increased with gestation but continued to increase in the third trimester, despite very little change in fat mass. However, this represents a similar increment in plasma leptin in both groups (approximately 35% in obese women vs 40% in lean women between 16 and 36 weeks). Obese women have significantly

greater fat mass than lean women, which could account for overall greater levels of leptin; however, obese women gain less fat mass during pregnancy than lean women, suggesting that increment in plasma leptin is not simply a function of increment in fat mass.

Misra and Trudeau also observed an increase in serum leptin with gestation in a cohort of normal weight and overweight/obese ($\text{BMI} > 26.0 \text{ kg/m}^2$) women, but found a smaller rate of increase for obese compared with lean women, and a significant reduction in serum leptin per kg body weight in obese but not lean women (Misra and Trudeau, 2011). In contrast, in our cohort, plasma leptin/kg body weight increased more with gestation in obese compared to lean women (0.28 ± 0.04 vs 0.33 ± 0.06 ng/ml/kg between 16 and 36 weeks in lean women, $n = 14$; 0.47 ± 0.08 vs 0.60 ± 0.07 ng/ml/kg between 16 and 36 weeks in obese women, $n = 12$; $p < 0.05$). However, when this was calculated using kg fat mass rather than total body weight, there was a small but not significant increase in obese women, and there were no significant differences between lean and obese women at any gestation, supporting the hypothesis that tissues other than adipose have a significant contribution to gestational increases in maternal leptinaemia in human pregnancy.

The relevance of such calculations is somewhat limited: while they may reflect a genuine upregulation of leptin production by tissues other than adipose in obese pregnant women, the values generated are influenced firstly by potential errors in estimation of fat mass in pregnancy and secondly by a larger volume of distribution in obese women, which renders estimation of leptin production inaccurate, as the same mass of leptin will correspond to a lower plasma concentration in subjects with a larger circulating volume. It is

therefore important to directly measure leptin secretion from tissues to more comprehensively assess the true rate of secretion.

In human pregnancy, placenta is a major source of leptin (Masuzaki, Ogawa *et al.* 1997), in contrast to other species such mouse, where placental leptin is not synthesized (Malik, Carter *et al.* 2005) and adipose tissue is the major source. Placental leptin transcript levels were significantly lower in term placenta compared with first trimester (Henson & Castracane, 2000), mirroring the pattern of circulating maternal leptin. Placental leptin is largely released into the maternal circulation (Linneman, Malek *et al.* 2000), and may have a direct role in orchestrating maternal metabolic responses to pregnancy. It may also be important in regulation of energy substrate supply to the fetus despite very little direct secretion to the fetal side of the placenta. Placental amino acid transport is influenced by maternal leptin concentrations: leptin influences system A amino acid uptake via a STAT3-dependent pathway (von Versen-Hoynck, Rajakumar *et al.* 2009), and is influenced by obesity: maternal hyperleptinaemia is associated with reduced placental system A amino acid transport, and reduced leptin-stimulated amino acid uptake by placenta in obese compared with lean women (Farley, Tejero *et al.* 2010).

The functional role of hyperleptinaemia in pregnancy is thought to be as part of the regulation of maternal appetite; in non-pregnant individuals, leptin has a central action to suppress appetite. In pregnancy, the development of central leptin resistance could account for the presence of maternal hyperphagia in the face of increasing maternal fat stores. In mice, leptin withdrawal at various stages in gestation of ob/ob mice did not affect

successful parturition but did influence maternal appetite and weight gain, (Mounzih, Qiu *et al.* 1998). In rats, direct intracerebroventricular (icv) infusion of leptin in pregnant animals fails to suppress maternal appetite, unlike in non-pregnant animals (Ladyman and Grattan, 2004), consistent with hypothalamic leptin resistance. There are increased levels of leptin-binding protein in pregnant rats, limiting the availability of leptin to the brain (Seeber, Smith *et al.* 2002). Downregulation of leptin receptor in the ventromedial hypothalamic (VMH) nucleus has also been observed in pregnant rats, along with reduced STAT3 phosphorylation in response to leptin in the same region (Ladyman & Grattan, 2004). In non-pregnant animals, leptin also acts on both pro-opiomelanocortin (POMC) neurone and neuropeptide-Y (NPY) neurones in the arcuate nucleus of the hypothalamus to regulate anorectic and orexigenic stimuli respectively. In pregnant rats, levels of POMC mRNA decline and NPY increase in the arcuate nucleus with advancing gestation, despite concomitant increases in circulating leptin, though leptin-stimulated STAT3 phosphorylation in this region is not affected by pregnancy (Ladyman, Tups *et al.* 2009). Thus, despite elevated peripheral leptin levels secondary to increased fat stores or placental secretion, pregnancy is a state of functional central leptin resistance, with region-specific regulation of response to leptin.

3.4.4.2. Adiponectin in obese pregnancies

Obesity is associated with reduced expression and secretion of adiponectin from adipose tissue (Arita, Kihari *et al.* 1999). Adiponectin has a role in insulin sensitisation and can reverse the insulin resistance associated with obesity in mice (Yamauchi, Kamon *et al.* 2001). In normal weight pregnant women, circulating total adiponectin levels are maintained in the first trimester but then decline to lowest levels in the third trimester (Cseh, Baranyi *et al.* 2004; Fuglsang, Skjaerbaek *et al.* 2005; Catalano, Hoegh *et al.* 2006; Eriksson, Löf, *et al.* 2010; Paradisi, Ianniello, *et al.* 2010). Our findings support this, with reduced plasma total adiponectin in lean women from 28 weeks. In obese pregnant women, we found lower plasma total adiponectin at all three time points compared with lean women, but this was only significant at 16 weeks; this concurs with the findings of Nien *et al.* who found a similar convergence between lean and overweight women (Nien, Mazaki-Tovi, *et al.* 2007). Adiponectin levels in pregnancy correlate with insulin sensitivity (Cseh, Baranyi *et al.* 2004; Catalano, Hoegh *et al.* 2006) as they do in non-pregnant individuals (Weyer, Funahashi *et al.* 2001), but appear to also have a role in some pathological conditions, with further reduction in subjects with gestational diabetes (Horosz, Bomba-Opon *et al.* 2011), and paradoxical elevation in pre-eclampsia (Ramsay, Jamieson *et al.* 2003).

Adiponectin exists in plasma in a trimeric, hexameric and multimeric (high molecular weight) form. It is thought that the high-molecular weight form is most specifically related to its insulin-sensitising activity (Fisher, Trujillo, *et al.* 2005). In common with our findings, Catalano *et al.* demonstrated a

reduction in lean women between early and late gestation in high molecular weight as well as total adiponectin (Catalano, Hoegh *et al.* 2006); however, we have shown that no such decrease occurs in morbidly obese women, and that HMWA levels are slightly higher throughout pregnancy in obese compared with lean women, though this is not statistically significant (Figure 3.9). Instead, the ratio between total and HMW adiponectin is consistently elevated throughout pregnancy in obese compared with lean women (Figure 3.9). Whether this is observed in this cohort of women as an effect of pregnancy or obesity is not certain although elevated HMW:total adiponectin levels are associated with a reduced risk of development of type II diabetes in women (Heidemann, Sun *et al.* 2008) and may represent an adaptation to counteract development of insulin resistance.

Adiponectin may also have a role in modulating placental function. Placenta is a source of adiponectin in pregnancy (Caminos, Nogueiras, *et al.* 2005); it is localised to syncytiotrophoblast (Chen, Tan, *et al.* 2005), and trophoblast insulin-stimulated amino acid transport is negatively regulated by adiponectin in a STAT3-dependent manner (Jones, Jansson *et al.* 2010). Thus an additional benefit of elevated adiponectin in obese pregnant women might be to limit placental amino acid transport which could otherwise promote excessive fetal growth.

3.4.4.3. Inflammatory mechanisms

Several studies have indicated that obesity in pregnancy is associated with worsening of maternal inflammatory status (Ramsay, Ferrell, *et al.* 2002; Roberts, Riley *et al.* 2011); our findings support this, with evidence of enhanced circulating levels of C-reactive protein, IL-6 and MCP-1. While there was no significant relationship between gestation and non-specific markers of inflammation such as CRP or specific cytokines such as TNF α , both MCP-1 and IL-6 were significantly increased in lean and obese subjects with increasing gestation, indicating a potential pregnancy specific effect. IL-6 and MCP-1 have been implicated in the development of insulin resistance in non-pregnant obese subjects, contributing to intra-adipose IR (Kern, Ranganathan *et al.* 2001; Bastard, Maachi, *et al.* 2002) and hepatic IR (Senn, Klover, *et al.* 2002; Klover, Zimmers, *et al.* 2003; Cai, Yuan, *et al.* 2006).

Increases in TNF α and IL-6 in normal pregnancy have previously been reported (Makhseed, Raghupathy *et al.* 2000; Curry, Vogel *et al.* 2008) and increased levels of TNF α have been associated with insulin resistance in pregnancy (Kirwan, Haugel-de-Mouzon, *et al.* 2002). In our cohort, there was an increase in TNF α in lean women between 16 and 28 weeks with a decline in the third trimester; in obese women, TNF α was modestly increased throughout pregnancy compared to lean women. Excess inflammatory markers in obese subjects in the third trimester may contribute to enhanced IR observed in obese patients at this gestation.

Features of intra-adipose inflammation include infiltration of macrophages (Weisberg, McCann, *et al.* 2003), a phenotypic switch in those macrophages to

a type 1 pro-inflammatory response (Lumeng, Bodzin, *et al.* 2007), as well as increased synthesis and secretion of pro-inflammatory cytokines eg. TNF α and IL-6 by both adipocytes and stromal vascular cells (Hotamisilgil, Arner, *et al.* 1995; Kern, Saghizadeh *et al.* 1995). It is likely that this system plays a role in both the response to increasing adiposity and as a contributor to the development of subsequent insulin resistance. Adipose tissue macrophages are observed in obese adipose tissue in crown-like structures surrounding apoptotic adipocytes (Cinti, Mitchell *et al.* 2005); increased MCP-1 synthesis is important for macrophage infiltration and activation (Kanda, Tateya, *et al.* 2006; Kim, Park *et al.* 2006). Several cytokines have direct action on insulin signalling in adipocytes: TNF α downregulates expression of the insulin receptor and IRS1; IL-6 reduces the expression of IRS-1 in 3T3-L1 cells, and reduces insulin stimulated glucose transport (Rotter, Nagaev, *et al.* 2003).

The role of the adipose tissue macrophage in the development of insulin resistance of normal pregnancy has not yet been clearly described. In normal mice, pregnancy induces infiltration of macrophages in adipose tissue in late pregnancy, with associated upregulation of CD68, MCP1, TNF α and PAI1 in subcutaneous adipose tissue (Zhang, Sugiyama *et al.* 2011). This may therefore indicate that macrophage infiltration is a normal response to episodes of adipose tissue expansion, with a role in the normal development of IR in pregnancy. While we observed an upregulation of IL-6 and MCP-1 in the subcutaneous adipose tissue of obese subjects, there was no relationship between BMI and transcript levels of CD68, a surrogate marker of mature macrophages. This may be a reflection of a number of factors. Firstly, transcript level of CD68 may not be the best indicator of the presence or absence of macrophages. Secondly, we correlated CD68 transcript levels with

BMI recorded at the booking visit in early pregnancy. Lean women undergo a significant increase in weight in pregnancy, and early pregnancy BMI will not be reflective of weight gain during pregnancy. Thus there may be a correlation between adipose CD68 transcript levels and third trimester BMI which was not apparent when comparing CD68mRNA and booking BMI.

The uteroplacental unit is a major source of pro-inflammatory cytokines (Denison, Kelly, *et al.* 1998; Lappas, Permezel *et al.* 2004), and is a potential significant contributor to gestational increases in circulating cytokines. In this study, in lean women, IL-6 and MCP-1 levels increase from 16 to 28 weeks, followed by a smaller, not statistically significant, rise to 36 weeks. However, in obese women, there is a further significant increase in IL-6 and MCP-1 between 28 and 36 weeks, leading to an enhanced pro-inflammatory environment in obese women at the end of the third trimester. This supports the findings of several previous studies at term which have demonstrated enhanced circulating pro-inflammatory cytokines (Ramsay, Ferrell, *et al.* Roberts, Riley *et al.* 2011). It may also suggest that the increase seen in early pregnancy is part of a normal response to pregnancy, but that further subsequent increases are the additional contribution of obesity. Notably, placental expression of pro-inflammatory cytokines is increased in obese compared with lean placentae in sheep and humans (Zhu, Du, *et al.* 2010; Roberts, Riley *et al.* 2011), along with increased macrophage density (Challier, Basu, *et al.* 2008).

The role of inflammation in normal pregnancy is complex. While increases in pro-inflammatory cytokines with advancing gestation have previously been reported as above, opposing anti-inflammatory processes are also thought to

be important. Indirect evidence for activation of anti-inflammatory pathways comes from improvement in several pro-inflammatory conditions during pregnancy, such as rheumatoid arthritis (Nelson, Hughes, *et al.* 1993; Hazes, Coulie, *et al.* 2011). However, not all inflammatory conditions are improved by pregnancy and it is clear that inflammatory processes still occur in pregnancy, such as acute pancreatitis, pelvic inflammatory disease and sepsis. In particular, pregnancy is associated with an increased susceptibility to sepsis and excess mortality from infections such as influenza (Neuzil, Reed, *et al.* 1998).

Immune suppression in pregnancy was postulated as a mechanism to explain lack of rejection of placental tissue expressing paternal antigen or rejection of the fetus by the maternal immune system; however, it is more likely that there is a complex phased interaction between the maternal immune system and trophoblast designed to facilitate implantation, support of a successful pregnancy and ultimately contribute to initiation of the inflammatory event of parturition (Mor, Cardenas, *et al.* 2012). Therefore, while there is immune modulation in pregnancy, it cannot be wholly viewed as an anti-inflammatory environment.

In pregnancy, there is a balance between pro-inflammatory and pro-resolution mechanisms, particularly in the context of initiation of parturition (Jabour, Sales, *et al.* 2009). These are thought to become disordered in some cases of preterm labour, particularly those triggered by infection, with upregulation of pro-inflammatory pathways in preterm labour (Romero, Gotsch, *et al.* 2007). In contrast, the pro-resolution cytokine interleukin-10 (IL-10) is increased in early pregnancy but is reduced at term, permitting onset of

the timed inflammatory process of labour (Hanna, Hanna, *et al.* 2000; Thaxton & Sharma, 2010).

A complex orchestration of cytokines helps to regulate the balance between sub-populations of T-helper cells necessary for pregnancy, where a shift towards a Th2 response is thought to favour a successful pregnancy (Ernerudh, Berg, *et al.* 2011); the observation that there is an increase in 'inflammation' or an increase in what are considered pro-inflammatory cytokines in this population of patients is therefore only one aspect of the cytokine profile of normal pregnancy. Excess inflammation observed in obese patients may contribute to an altered immunological environment, which in early pregnancy may account for reduced fertility and by later in gestation, difficulties in initiation and propagation of labour.

3.4.5. Consequences of enhanced insulin resistance in obese pregnant women

One of the main functions of adipose tissue is storage of energy in the form of triglyceride. Uptake of circulating fatty acids into adipocytes occurs via fatty acid transport proteins (FATPs); translocation of these proteins to the cell membrane can be regulated by insulin (Wu, Ortegon, *et al.* 2006). Glucose uptake into adipocytes occurs via GLUT4, also in an insulin-dependent manner. Insulin acts to suppress the opposing pathway of lipolysis via inhibition of hormone-sensitive lipase. In insulin-resistant states therefore, uptake of fatty acids is reduced, and release of fatty acids and glycerol enhanced.

In pregnancy, elevated circulating free fatty acids in late pregnancy has long been recognised (Warth, Arky, *et al.* 1975). Enhanced lipolysis in the third trimester has also been described (Diderholm, Stridsberg, *et al.* 2005), and correlated with birth weight (Diderholm, Stridsberg, *et al.* 2006). The increase in plasma NEFA that we have observed in our lean cohort at 36 weeks is consistent with these previous observations. Similarly, enhanced insulin resistance in obese subjects is consistent with elevated NEFA throughout pregnancy (Figure 3.10). The relative decrease in NEFA observed between 16 and 28 weeks in obese subjects may reflect increased uptake and utilisation of NEFA in response to the energy requirements of late pregnancy, despite maintenance of an insulin-resistant maternal environment.

Under physiological conditions in fasted individuals, reduced insulin stimulation promotes release of NEFA and glycerol from adipose tissue and

drives uptake by liver during fasting as a substrate for gluconeogenesis. In the context of insulin resistance, intra-adipose uptake of NEFA is reduced, leading to spillover into ectopic sites such as liver or skeletal muscle without utilisation via beta-oxidation. In non-pregnant, obese individuals, plasma NEFA levels correlate with the degree of hepatic steatosis, with greater steatosis in subjects with higher NEFA (Holt, Wild, *et al.* 2006). In obese pregnant women, pre-existing insulin resistance and saturation of adipose tissue storage capacity may contribute to enhanced hepatic steatosis.

While not specific for fatty liver, mild elevation of liver aminotransferases such as ALT can be an indicator of hepatocellular damage and is a feature of hepatic steatosis. We have observed mild elevation of ALT in obese pregnant women, with significant elevation compared with lean women in early pregnancy (Figure 3.11), followed by a subsequent reduction with increasing gestation. This unexpected finding may reflect improvement of hepatic steatosis in obese women in pregnancy, despite severe insulin resistance in maternal tissues and elevated plasma NEFA. This may be a consequence of preferential diversion of circulating fatty acids to the fetus in late pregnancy. These data are partly in keeping with the study by Meyer *et al.* who found that obese women began pregnancy with greater plasma triglyceride levels compared with lean women but reached a similar maximum level in the third trimester; however, they did not find a similar pattern of change in plasma NEFA, which may be a reflection of less severe obesity in their cohort (Meyer, Stewart, *et al* 2013).

3.4.6. Adipose tissue lipid storage

In obesity, adipose tissue lipid accumulation is increased, which may be secondary to re-esterification of dietary triglyceride in adipose tissue as well as de novo lipogenesis. One of the key steps of de novo lipogenesis is regulated by fatty acid synthase (FASN). This process is not normally considered to contribute significantly to overall lipogenesis, but has previously been observed to be upregulated in obesity and insulin resistance (Berndt, Kovacs *et al.* 2007) and thus thought to contribute to lipid accumulation in obesity, particularly in the visceral compartment. In contrast, we have observed a negative correlation between BMI and transcript levels of FASN in subcutaneous and omental adipose tissue in the third trimester (Figure 3.12). In the third trimester, maternal adipose stores are consumed to supply the energy requirement of the fetus: de novo lipogenesis in adipose tissue would not be desirable in this context. Transcript levels of other genes involved in the lipogenic pathways were not correlated with BMI in this cohort. This points to a possible pregnancy specific mechanism facilitating diversion of lipid from adipose tissue to the fetus, despite the opposing stimulus secondary to obesity. However, there is also conflicting evidence that adipose de novo lipogenesis is reduced in the context of obesity, and more specifically, where there is reduced insulin sensitivity (Diraison, Dusserre, *et al.* 2002; Ranganathan, Unal *et al.* 2006). Therefore, lower levels of FASN in correlation with higher BMI may be a reflection of the degree of insulin resistance in obese subjects.

3.5. Conclusions

We have shown that obese pregnant women have significant perturbation of lipid and carbohydrate metabolism, with evidence of enhanced insulin resistance throughout pregnancy, consequent elevation of plasma NEFA, and diversion from the pregnancy-related changes in adipokines and inflammatory profile seen in lean pregnant women. However, despite the dual metabolic insult of obesity and pregnancy, the divergence between the two groups was not as pronounced as initially hypothesised, and many indices appeared to converge rather than diverge eg. plasma adiponectin profile.

The contribution of adipose tissue to this pattern is likely to be less significant than initially hypothesised. While obese pregnant women have a much greater adipose tissue mass compared with lean women, pregnancy-related expansion of this fat mass appears to be significantly curtailed. At term, the transcript profile of adipose tissue is broadly similar between lean and obese women, and the characteristic responses observed in non-pregnant obese subjects are noticeably absent eg, lack of upregulation of leptin. This points to discrete mechanisms driving the metabolic changes of pregnancy and obesity, despite many similarities in the physiological consequences of these conditions. In obesity, overexpansion of adipose tissue appears to generate a local inflammatory response and local dysfunction of insulin regulation in this site, which then spills over systemically to influence hepatic and skeletal muscle insulin regulation, culminating in a vicious cycle of systemic metabolic dysregulation. In pregnancy, the profound but ultimately reversible changes in maternal

metabolism appear to co-opt some of these mechanisms, particularly those involving inflammatory cytokines, and adipokines. Dissection of these mechanisms remains difficult and their interactions even more so, though placenta is implicated partly because of the relatively rapid resolution of normal maternal metabolism following delivery and because of discordance in temporal changes in adipokines and inflammatory markers between lean and obese women which are not explicable by changes in fat mass alone.

Our analysis of HOMA and QUICKI in this chapter showed that obese pregnant women have a poorer metabolic profiles (in terms of greater insulin resistance/ decreased insulin sensitivity) compared with lean pregnant women in the third trimester. We wanted to define whether this was related to hepatic or peripheral insulin resistance, and to confirm that these differences persist when examined using the gold standard hyperglycaemic euglycaemic clamp. Thus we recruited a cohort of obese and a comparator cohort of lean women to undergo HEC studies at various time points during pregnancy. The results are shown in the next chapter.

Chapter 4

Whole body insulin sensitivity and lipolysis: hyperinsulinaemic euglycaemic clamp studies

4.1. Background

Obesity and pregnancy both lead to profound changes in the regulation of metabolism. In the previous chapter, we have shown that obese pregnant women had evidence of deranged metabolism and excess inflammation throughout pregnancy compared with lean pregnant women. However, there were also some features which are not as divergent from the lean phenotype as initially hypothesised, such as the transcript characteristics of adipose tissue at term. It was not possible to dissect which of these features were secondary to the effects of pregnancy and which were secondary to obesity ie. were the lean women becoming more 'obese' in their phenotype, or was there a 'normalisation' effect of pregnancy in obese women? In this chapter, a longitudinal case-control study was designed which included a non-pregnant control group to examine some aspects of intermediary metabolism and the response of adipose tissue with the aim of separating the relative contributions of obesity and pregnancy.

Central to the metabolic response to both obesity and pregnancy is modification of insulin sensitivity in liver, adipose tissue and skeletal muscle. Insulin sensitivity can be assessed by a number of means (see Chapter 1), but the gold standard method of assessment of whole body insulin sensitivity is the hyperinsulinaemic euglycaemic clamp (HEC). This technique involves a continuous infusion of insulin to achieve supraphysiological plasma

concentrations with a concomitant infusion of glucose to maintain euglycaemia at approximately 4.7 mmol/L. Insulin-stimulated glucose uptake under steady state conditions is used as a measure of IS and is calculated from the amount of glucose required to maintain euglycaemia in such conditions. The use of stable isotope tracers in the context of a HEC allows further detail of regulation of insulin sensitive pathways to be defined, including endogenous glucose production and rate of lipid breakdown.

Insulin sensitivity (IS) is often reduced in obese subjects, which contributes to the pathogenesis of Type II diabetes mellitus, (De Fronzo and Ferrannini, 1991). Where there is reduced IS, metabolic pathways normally opposed by insulin are freed from inhibition. Consequently, lipolysis is enhanced, leading to elevated release of free fatty acids (FFAs) and glycerol, particularly from visceral adipose tissue (Nielsen, Guo *et al.*, 2004). In turn, this leads to increased circulating fats and a lipotoxic effect on a number of organs, notably liver (Boden, Cheung *et al.*, 2002) and skeletal muscle (Liu, Jahn *et al.*, 2011) which further exaggerates insulin resistance at these sites.

In pregnancy, IS decreases in lean women by approximately 60% between early and late gestation (Catalano, Tyzbir *et al.*, 1991). This fall in IS is associated with enhanced lipolysis in the third trimester (Diderholm, Stridsberg *et al.*, 2005). This change with gestation has been proposed as an evolutionary mechanism to promote energy storage initially while pregnant women are insulin sensitive, followed subsequently by diversion of maternal dietary fuels from storage in maternal depots to be used for offspring growth in late pregnancy and during breastfeeding. The cellular mechanisms

underlying this are not fully understood, but are likely to involve post-receptor defects in insulin-sensitive tissues such as downregulated IRS-1 in skeletal muscle (Barbour, McCurdy *et al.*, 2007). The factors underlying decreased IS are not clearly established, although a complex interaction between placental and maternal hormones seems likely.

The adverse consequences of enhanced lipolysis and excess free fatty acid release outwith pregnancy include elevated plasma lipids, endothelial damage, atherogenesis and consequent cardiovascular disease. In pregnancy, a degree of enhanced lipolysis is not only desirable, it is essential for normal growth: reduced third trimester lipolysis is associated with growth restriction (Diderholm, Stridsberg, *et al.*, 2006). The major pregnancy-related morbidities in obese women include gestational diabetes, pre-eclampsia and increased birthweight of offspring. Obesity-related metabolic changes may contribute to the pathogenesis of these conditions as a result of enhanced insulin resistance, FFA-associated endothelial damage and increased nutrient supply to the fetus. However, with respect to lipolysis, the specific changes in insulin sensitivity of severely obese non-diabetic pregnant subjects has not yet been described.

The aims of this study were firstly, to characterise whole-body insulin sensitivity and rate of lipolysis in severely obese non-diabetic pregnant women and secondly, to identify potential drivers of these metabolic adaptations.

We hypothesised that:

- Severe obesity in pregnancy is associated with exaggerated insulin resistance in the third trimester as assessed by use of the hyperinsulinaemic euglycaemic clamp.
- Worse IR is associated with reduced endogenous hepatic glucose production and enhanced lipolysis.
- Changes in insulin sensitivity are associated with changes in transcript level of genes regulating inflammation, lipolysis and lipogenesis in subcutaneous adipose tissue at an earlier gestation, with a convergent pattern of expression in the third trimester.

4.2. Methods

4.2.1. Study Title and Ethical Approvals

Origins and Consequences of Altered Metabolic Processes in Obese Pregnant Women (AMPOP).

Chief Investigator Professor Jane Norman

Principal Investigator Dr Sarah Barr

Ethical approval was sought from Lothian Research and Ethics Committee in January 2009. Approval was gained in April 2009 (LREC number 09/S1103/6, 9 April 2009). This study was co-sponsored by NHS Lothian R&D (R&D number R/RM/2009/04, April 2009). Five substantial amendments were approved (1, September 2009; 2, November 2009; 3, January 2010; 4, June 2010; 5, September 2010). The study was carried out at the Wellcome Trust Clinical Research Facility at the Royal infirmary of Edinburgh.

4.2.2. Overview

Women were recruited in 4 groups:

Obese pregnant	OP
Obese non-pregnant	ONP
Lean pregnant	LP
Lean non-pregnant	LNP

Obesity was defined as BMI $> 35\text{kg/m}^2$ to ensure an adequate population of women with severe obesity from which to recruit. While obese pregnant patients were drawn from a population with booking BMI $> 40\text{kg/m}^2$, non-pregnant obese patients remained adequately matched for BMI (Table 4.1). Lean participants were defined as having BMI $20.0 - 24.9 \text{ kg/m}^2$. Pregnant participants were asked to undergo two clamp procedures, at approximately 19 weeks gestation ('early' pregnancy, E) and at approximately 36 weeks gestation ('late' pregnancy, L). All non-pregnant participants underwent a single clamp procedure. Prior to all clamp procedures, a needle biopsy of abdominal subcutaneous adipose tissue was obtained.

Pregnant participants were recruited from routine antenatal clinics in Lothian and from the Metabolic Antenatal Clinic previously described. Non-pregnant volunteers were recruited following response to advertisement, women who had previously attended the Metabolic Antenatal clinic and had given permission to be contacted about further studies, and from the bariatric surgery clinic at the Royal Infirmary of Edinburgh.

Seventy four eligible pregnant women were given study information between July 2009 and March 2011; of these, 19 women attended for screening (12 obese and 7 lean).

Advertisements were placed for non-pregnant participants in February 2010, March 2010, February 2011 and March 2011; 65 eligible respondents were given study information. A further 35 women who had previously attended the Metabolic Antenatal clinic and had given permission to be contacted about further studies were also sent study information. Sixteen potential non-pregnant participants attended for screening (9 obese and 7 lean).

4.2.3. Inclusion criteria, exclusion criteria and pre-clamp screening

Participants were eligible for inclusion if they were aged 18-45 years, healthy and of Caucasian ethnic origin. Exclusion criteria included hypertension, diabetes, active endocrine disorders (including thyroid hormone replacement), polycystic ovarian syndrome, previous history of gestational diabetes, use of anti-inflammatory or glucocorticoid medications (including inhaled or topical). These criteria were chosen to exclude women with conditions or features known to have an independent effect on insulin sensitivity. Where possible, non-pregnant participants had regular menstrual cycles; clamps were carried out in the follicular phase of the cycle, determined by the date of the last menstrual period. Oral contraceptive medication was not considered an exclusion; clamps were carried out in the pill-free week where applicable. Three patients were using continuous progesterone preparations as contraception. Specific exclusion criteria for pregnant women included multiple pregnancy, gestational diabetes, pre-eclampsia, large-for-gestational age or small-for-gestational age fetus.

All non-pregnant subjects underwent a 75g oral glucose tolerance test prior to participation. WHO guidelines were used to exclude current diabetes: fasting plasma glucose $<7.0\text{mmol/L}$, and 2-hour plasma glucose $<11.1\text{mmol/L}$. All pregnant subjects had a fasting plasma glucose sample prior to participation and a 75g oral glucose tolerance test at 28 weeks gestation. SIGN criteria were used to exclude GDM: fasting plasma glucose $<5.5\text{mmol/L}$, and 2-hour plasma glucose $<9.0\text{mmol/L}$. Where additional risk factors for GDM were present, eg. obesity or previous macrosomia, then an oral glucose tolerance test was carried out instead of a fasting glucose alone

prior to participation. All subjects also had fasting plasma (EDTA and lithium heparin anticoagulants) and serum samples obtained for analysis of cytokines, FFAs and adipokines. The samples were collected, chilled, separated and stored as described in Chapter 2 (sample handling).

The height and weight of each participant was recorded as well as waist, hip, mid-upper arm and mid-thigh circumferences. Biceps, triceps and subscapular skinfold thicknesses were measured using Harpenden callipers and used to assess body fat distribution. Fat mass was estimated by bio-electrical impedance. Pregnant patients had anthropometry carried out at each visit.

4.2.4. Materials

For materials, including drugs and tracer details, see Chapter 2.

Baseline fasting lipid profiles (including total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and triglyceride), plasma glucose, liver function tests, and urea and electrolytes (sodium, potassium, chloride, bicarbonate and creatinine) were measured at the biochemistry laboratory of the Royal Infirmary of Edinburgh.

The following tasks were carried out by the candidate: all subject recruitment; glucose tolerance testing; adipose tissue biopsies; all clamp studies; plasma cytokine and adipokine quantification by ELISA; RNA extraction, cDNA synthesis and real time RT-PCR (see Chapter 2 for details of protocols).

Stable isotope enrichment was measured using gas-chromatography mass spectrometry by Sanjay Kothiya in the Wellcome Trust Mass Spectrometry Core Facility as detailed below (and see Chapter 2 for protocol). Serum insulin and NEFA were analysed by Graham Harold (senior technician) as detailed in Chapter 2.

4.2.5. Adipose Tissue Biopsy

Participants attended at 0730h following an overnight fast. A subcutaneous adipose tissue biopsy was carried out under aseptic conditions. Patients were positioned supine, abdominal skin exposed, skin cleaned with chlorhexidine and draped. 1% lignocaine without adrenaline was used to anaesthetise an area of skin and subcutaneous tissue approximately 5cm² lateral to umbilicus. Using a 12G needle and 50mL syringe, adipose tissue was aspirated under suction. Adipose tissue was then quickly rinsed with sterile saline to remove obvious blood contamination, transferred to a 2mL round-bottom eppendorf, labelled and placed immediately on dry ice. Two to three aspirations were carried out to ensure sufficient material for analysis was obtained. Pressure was applied to the site to prevent bruising for 5 minutes following aspiration; the site was then cleaned and covered with a dressing. For biopsies in pregnant women, abdominal examination was carried out prior to the procedure to identify the uterine fundus. At 36 weeks, women were positioned in a left lateral position to prevent aorto-caval compression.

RNA extraction, cDNA synthesis and real time RT-PCR was carried out as described in Chapter 2. Briefly, tissue in 1mL qiazol lysis solution was homogenised using steel beads with a TissueLyser for 2 × 3 minutes at 25Hz. RNA was purified from the homogenate following chloroform and ethanol extraction using columns and resuspended in RNase-free H₂O (Qiagen Lipid MiniKit). On-column DNase digestion was carried out. RNA was quantified spectrophotometrically and stored at -80°C until use. RNA (500ng) was reverse transcribed using a commercial cDNA synthesis kit (Applied Biosystems). All PCR primers and probes were obtained from Applied Biosystems as ready-made, optimised gene expression assays (for list of

targets, see Chapter 2). Quantitative real time RT-PCR was carried out using Taqman® reagents and the 7900HT Fast Real-Time PCR system. A standard curve was generated for each gene using serial dilutions of pooled cDNA from all samples. All samples were assayed at either 1:10 or 1:20 dilutions, according to the relative abundance of the gene of interest. Transcript levels were normalised to levels of either 18S or cyclophilin A.

4.2.6. Hyperinsulinaemic Euglycaemic Clamp

4.2.6.1. Participant preparation

Clamp procedures were carried out following adipose biopsy. A 17G cannula was inserted into a peripheral arm vein for administration of insulin, dextrose and tracer solutions via a multichannel adaptor. Baseline plasma and serum samples were obtained for determination of baseline tracer enrichment, fasting plasma glucose and fasting lipid profile respectively. A second 17G cannula was inserted into a distal vein in the contralateral arm for blood sampling. This arm was wrapped in a heated blanket for the duration of the study. A slow IV infusion of 0.9% saline was infused through this cannula to maintain cannula patency.

4.2.6.2. Drug preparation & dosage calculation

Actrapid insulin was prepared in 0.9% saline at a concentration of 0.3U/mL. Insulin was infused at 20mU/m²/min (low dose) and 40mU/m²/min (high dose), according to the body surface area (BSA) in m² of the subject. BSA was calculated using the Mosteller formula:

$$BSA (m^2) = \sqrt{\frac{weight (kg) \times height(m)}{3600}}$$

The rate of infusion in mL/min of stock insulin solution was calculated as follows:

$$Rate (ml/min) = \frac{(20 \text{ or } 40) \times BSA \times 60}{1000 \times 0.3}$$

Stock solutions of stable isotope tracers were prepared by Dr Alistair Millar, Radiopharmacist, Royal Infirmary of Edinburgh, using water as diluent. 1,1,2,3,3-²H₅-glycerol was provided in 8.5mL vials at a concentration of

40mg/mL; 6,6-²H₂-glucose was provided in vials of 5.0mL at a concentration of 350mg/mL.

Both tracers were prepared on the day of study for use in a single infusion volume of 300mL of 0.9% saline and infused at a rate of 50ml/hour.

The molar weight of d5-glycerol is 97g/mol; molar weight of d2-glucose is 182g/mol.

D5-glycerol was prepared at a concentration of 0.132μmol/kg/ml to achieve a delivery rate of 6.6 μmol/kg/hour; d2-glucose was prepared at a concentration of 0.44μmol/kg/ml to achieve a delivery rate 22 μmol/kg/hour.

Infusion was prepared as follows in a final volume of 300ml:

D5-glycerol: 0.123μmol/kg/ml = 12.8μg/kg/ml = 3.84mg/kg in 300ml;

D2-glucose: 0.44μmol/kg/ml = 80μg/kg/ml = 24mg/kg in 300ml.

4.2.6.3. Clamp Protocol

A bolus dose of d5-glycerol ($1.6 \mu\text{mol/kg} = 155.2 \mu\text{g/kg}$) and d2-glucose ($25 \mu\text{mol/kg} = 4.5 \text{ mg/kg}$) was administered at time 0 mins, followed by continuous infusion of prepared tracers (as above) for 5.5 hours. At 60, 70, 80 and 90 minutes, plasma samples were obtained. All plasma samples were collected using lithium heparin anticoagulant. Samples were collected on ice, separated by centrifugation and plasma stored at -80°C as described in Chapter 2.

At 90 minutes, insulin infusion at $20\text{mU/m}^2/\text{min}$ was commenced for 120 minutes with concomitant variable infusion of 20% glucose. A loading regime for insulin infusion was used as follows:

90-95 minutes	(from start) 2 x low dose infusion rate
95-100 minutes	1.5 x low dose infusion rate
100 minutes onward	1 x low dose infusion rate

Blood samples were obtained every 5 minutes by first withdrawing approximately 5 mL blood from the vein as well as saline present in the adaptor, discarding this sample, then withdrawing approximately 0.3mL blood for sampling; whole blood glucose concentrations were measured using an AccuChek blood glucose monitor. The sampling cannula was flushed with 2mL 0.9% saline after each withdrawal. The rate of infusion of 20% glucose was titrated to maintain whole blood glucose concentrations between 4.5mmol/L and 5.5 mmol/L. Additional plasma samples were obtained every 30 minutes from 90 minutes using fluoride oxalate

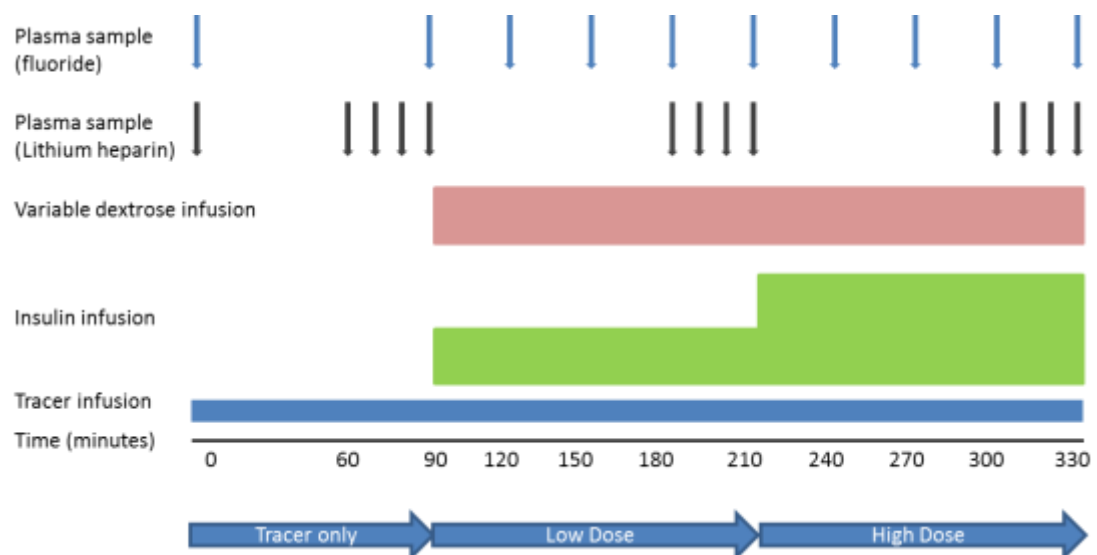
anticoagulant for assessment of plasma glucose. These analyses were carried out in the laboratory of the Royal Infirmary of Edinburgh.

At 180, 190, 200 and 210 minutes, plasma samples were obtained. At 180 minutes, insulin infusion rate was increased to 40mU/m²/min. At 300, 310, 320 and 330 minutes, plasma samples were obtained.

The volume of dextrose infused was noted at 180, 210, 300 and 330 minutes. These values were used to calculate the mean volume of dextrose required to maintain euglycaemia in steady state conditions in and thus the rate of glucose infusion at steady state in mg/kg/min (Glucose Infusion Rate, GIR).

A summary of the protocol is shown (Figure 4.1):

Figure 4.1 Summary of clamp protocol



4.2.7. Gas-Chromatography Mass Spectrometry

The GCMS protocol is detailed in Chapter 2. Briefly, 200 μ L plasma samples with 25 μ L internal standard mix ($^{13}\text{C}_6$ -Glucose and butanetriol) were deproteinised using 1mL acetonitrile and dried under nitrogen following vacuum filtration. Samples were then derivatised using pyridine and acetic anhydride (1:1 ratio) to form glucose pentacetate and glycerol triacetate, dried down under nitrogen at 37°C and reconstituted in 5% acetic anhydride. 1 μ L of the reconstituted solution was injected into the GCMS system as detailed in Chapter 2.

The peak abundance of ions for derivatised glucose (m/z 287), d2-glucose (m/z 289), glycerol (m/z 217), d5-glycerol (m/z 222) and internal standards $^{13}\text{C}_6$ -glucose (m/z 293) and butanetriol (m/z 231) were measured. Tracer/tracee ratios were calculated from the ratio of the peak areas and calibrated against standard curves of known concentration and isotopic enrichment. Sample measurements were corrected for background enrichment (mass+5 glucose and mass+2 glycerol present in baseline sample, prior to exogenous infusion of isotopes).

4.2.8. Calculations

Mean glucose disposal (M) under steady state conditions was calculated in mg/kg/min according to the volume of glucose infused over the last 30 minutes of each stage of the clamp study. This is the same as glucose infusion rate, (GIR) (Section 4.2.6). This is expressed per mg total body weight as it is a measure of whole body glucose disposal. Total body weight was used to express glucose disposal to account for whole body utilisation of glucose. Although expression of glucose utilisation per kg fat-free mass is also used in the literature and is likely to include the contribution by the uteroplacental unit, a whole body measure of glucose utilisation will also include insulin-stimulated glucose uptake by adipose tissue, which in obese subjects, contributes significantly to total body weight. An index of insulin sensitivity was also derived (M/I) which was calculated by dividing M by the mean insulin concentration (mU/L) during that part of the clamp.

Rate of appearance (Ra) and rate of disappearance (Rd) glucose and glycerol were calculated as follows:

Firstly, tracer: tracee ratio (T/Tr) was calculated for each time point using the peak areas on the mass spectrum corresponding to each isotope. Tracee refers to unlabelled glucose or glycerol and tracer refers to labelled glucose or glycerol.

Ra glucose was calculated as follows:

$$\frac{\text{Tracer}}{\text{Tracee}} = T/Tr = \frac{Ra \text{ d2glucose}}{Ra \text{ glucose}}$$

Therefore:

$$Ra \text{ Glucose} = \frac{Ra \text{ d2Glucose}}{T/Tr}$$

The rate of appearance of labelled glucose and glycerol are known as this corresponds to the rate of tracer infusion:

$$\begin{array}{llll} \text{Ra d2glucose} & 4\text{mg/kg/hour} & = & 0.067\text{mg/kg/min} \\ \text{Ra d5glycerol} & 0.64\text{mg/kg/hour} & = & 0.01\text{mg/kg/min} \end{array}$$

Ra glycerol is calculated in the same way, using the tracer:tracee ratios derived from the sample spectra. Rate of lipolysis can be calculated on the assumption that breakdown of triglyceride is the only source of unlabelled glycerol ie. Ra glycerol = rate of lipolysis.

Both Ra glucose and Ra glycerol are then corrected for and expressed per kg fat-free mass using fat-free mass estimates derived from bioimpedance assessments. Ra (glucose or glycerol) is equal to Rd (glucose or glycerol) when in isotopic steady state; Rd values represent substrate uptake by tissues. Expressing Rd per kg fat-free mass has the advantage of expressing substrate uptake according to the mass of tissue which utilises it, which is predominantly skeletal muscle. However, the disadvantage is that estimates of fat-free mass in pregnancy are potentially inaccurate due to the contribution of fetoplacental tissues (1.1.2.1, page 9) and does not take into account insulin-stimulated glucose uptake by adipose tissue, which contributes significantly to total body mass, particularly in obese subjects.

The natural abundance of other isotopic species in both the d2-glucose and the variable glucose infusion require further corrections to be made to the value used for Ra d2-glucose and GIR.

Firstly, the true Ra d2-glucose is composed of both d2-glucose present in the continuous infusion of tracer as well as background d2-glucose present in the variable glucose infusion. A correction of 92.5% was applied to the rate of infusion of tracer which reflects the true abundance of pure d2-glucose in the continuous infusion. The remainder of the glucose present in the continuous infusion is made up of other glucose species arising due to the natural abundance of other isotopes such as ¹³C. This leads to the formation of glucose molecules with additional mass, depending on how many of the 6 carbon molecules in glucose are replaced by heavier isotopes. Including all theoretical isotopologues, this means that only 92.5% of the mass of a preparation of d2-glucose is truly of this mass, which is important for quantification using GC-MS.

This isotopic distribution also applies to the variable glucose infusion, meaning that 1.1% of the variable GIR is composed of d2-glucose which is then added to the known rate of stable isotope infusion to obtain the true infusion rate of d2-glucose.

Therefore:

$$\begin{aligned}\text{True Ra d2-glucose} &= 92.5\% \text{ rate of infusion of d2-glucose} + 1.1\% \text{ variable GIR} \\ &= (0.925 \times 0.067) + (0.011 \times \text{GIR}) \\ &= 0.062 + (0.011 \times \text{GIR}).\end{aligned}$$

Secondly, steady state values for calculated Ra glucose were used to determine the rate of endogenous glucose production (EGP). Ra glucose is composed of both EGP as well as unlabelled glucose infused with the variable GIR. However, GIR must first be corrected for the true proportion of

unlabelled glucose present, which is also 92.5%, with the remainder being composed of other mass isomers, including d2-glucose and ¹³C substitutions. EGP is then calculated by subtracting the rate of corrected variable GIR from calculated Ra glucose:

$$\text{EGP} = \text{Ra glucose} - (0.925 \times \text{GIR})$$

No exogenous unlabelled glycerol was infused and the abundance of other isotopic species within the tracer infusion is sufficiently low as to be negligible; therefore no correction was required to the value of Ra glycerol.

4.2.9. Statistical analyses

Data are expressed as mean \pm SEM unless otherwise stated. Specific between-group comparisons of descriptive characteristics of the cohort were made using unpaired Student's t-test unless otherwise stated.

Data from the hyperinsulinaemic euglycaemic clamps, and measurements of plasma and adipose tissue variables were analysed using 2-way analysis of variance. Bonferroni post-hoc testing was used to detect between-group differences using GraphPad Prism software. Significance was set at $p < 0.05$. All statistical analyses were carried out by the investigator (SB).

Baseline values for weight were used other than tracer and insulin dosage at visit 1 and GC-MS calculations. Mean weight change between baseline and visit 1 for all subjects was 0.6 ± 0.1 kg.

Data are presented for all participants with satisfactory screening who went on to attend Visit 1 (16 weeks gestation). Pregnant subjects who were diagnosed with gestational diabetes at 28 weeks were excluded from participating in Visit 2 (36 weeks gestation: $n = 1$, lean group; $n = 3$, obese group). Their data from Visit 1 was included in analysis as they had had normal glucose tolerance testing prior to entering the study and were not diabetic at the time of Visit 1. Two otherwise eligible pregnant women were unable to attend for Visit 2, one lean and one obese. Final cohort numbers are as follows: Lean non-pregnant, $n = 7$; Obese non-pregnant, $n = 7$; Lean pregnant Visit 1, $n = 6$; Lean pregnant Visit 2, $n = 4$; Obese pregnant Visit 1, $n = 9$; Obese pregnant Visit 2, $n = 5$.

We were unable to perform an *a priori* power calculation prospectively due to insufficient available data in obese women – we have done a post hoc power calculation was performed as follows.

We had anticipated that the difference between the M value in the obese and the lean might be maintained by the end of pregnancy, in other words that the M value in the obese would be 50% that in the lean. Our study had 80% power to show these differences at the 5% significance level, if they truly existed. In practice, we showed that the mean (SD) M value at term was 3.97 (1.118) in the lean and 2.84 (1.488) in the obese group; in other words, that the value in the obese was 71% that in the lean. A sample size of 22 in each group would have been required to give 80% power show that these differences were statistically significant at the 0.05 significance level.

We also showed a trend to a greater insulin stimulated lipolysis in the 36 week pregnant group. These differences were not statistically significant – a sample size of 8 in each group would have been needed to identify that the values we observed in the obese group were significantly greater than the lean, at the 5% significance level.

4.3. Results

4.3.1. Patient Baseline Characteristics

Demographic characteristic of participants are shown in Table 4.1. Details are given for all patients who had satisfactory screening and went on to attend their first clamp study, regardless of later exclusion/withdrawal.

There were statistically significant between-group differences in participant ages. Lean pregnant women were older than lean non-pregnant participants and obese pregnant women; obese pregnant women were younger than obese non-pregnant women. The groups were otherwise well matched in height, weight, and for pregnant participants, gestation at delivery and birthweight.

The proportion of nulliparous women was greater in the lean non-pregnant group compared to lean pregnant (7/7 versus 1/6, Fisher's exact-test, $p = 0.005$); the proportion of nulliparous women in the lean non-pregnant group was also significantly greater compared to the obese non-pregnant group (7/7 versus 2/7, Fisher's exact-test, $p = 0.02$).

There were no significant differences in gestation at each visit between lean and obese pregnant groups.

Table 4.1 Demographic Characteristics of Study Cohort

	Pregnant			Non-pregnant		
	Lean (n=6)	Obese (n=9)	p ^a	Lean (n=7)	Obese (n=7)	p ^b
Age (years)	35 ± 0.7	27 ± 1.2	0.0003	26 ± 0.7 ^c	34 ± 2.0 ^d	0.002
Height (m)	1.67 ± 0.02	1.66 ± 0.02	ns	1.63 ± 0.02	1.63 ± 0.02	ns
Weight (kg)	63.7 ± 1.9	118.0 ± 3.1	<0.0001	63.0 ± 3.1	109.8 ± 3.9	<0.0001
BMI (kg/m ²)	23.7 ± 0.4	42.6 ± 1.7	<0.0001	22.5 ± 0.6	41.2 ± 1.5	<0.0001
Parity (Nulliparous/ Total n)	1/6	4/9	ns	7/7 ^e	2/7 ^f	0.02
SBP (mmHg)	122.3 ± 7.2	119.3 ± 2.9	ns	122.1 ± 2.3	120.4 ± 4.5	ns
DBP (mmHg)	74.2 ± 3.4	77.3 ± 1.5	ns	72.9 ± 1.7	74.4 ± 3.7	ns
Mean gestation (weeks + days)						
Baseline	17+4	16+0	ns	-	-	-
Visit 1	19+3	19+0	ns			
Visit 2	36+1(n=4)	35+3 (n=5)	ns			
Mode of delivery						
SVD	4	4				
LUSCS	2	5		-	-	-
Total	6	9	ns			
Mean gestation at delivery (weeks + days)	40+2	40+0	ns	-	-	-
Mean birthweight(g)	3647 ± 140.9	3720 ± 148.5	ns	-	-	-

Data are expressed as mean ± SEM. ns = not significant. Blood pressure and weight given were measured at visit 1.

Details are given for all patients who had satisfactory screening and went on to attend first clamp study, regardless of later exclusion/withdrawal.

Comparisons were made between the following groups using unpaired Student's t-test to compare age, height, weight, BMI, SBP, DBP, gestations and birthweight; Fisher's exact test was used to compare parity and mode of delivery

- a Lean pregnant versus obese pregnant
- b Lean non-pregnant versus obese non-pregnant
- c p < 0.0001 lean pregnant versus lean non-pregnant
- d p = 0.007 obese pregnant versus obese non-pregnant
- e p = 0.005 lean pregnant versus lean non-pregnant
- f not significant, obese pregnant versus obese non-pregnant

4.3.2. Body composition

Changes in total body weight and estimated total body fat mass between baseline visit and study visit 2 are shown in Table 4.2. Fat mass was estimated using bioelectrical impedance (Tanita body composition analyser, see Chapter 2 for details). Limitations of the use of bioelectrical impedance for estimation of fat mass in pregnancy are discussed in Chapter 3.

Obese women were heavier than lean women as expected, both in the pregnant and non-pregnant groups. There was an increase in weight from baseline to visit 2 in both lean and obese pregnant women but this was not statistically significantly different between the two groups. However, when weight gain was expressed as a percentage change from baseline, lean women had a greater percentage increase in weight compared with obese women.

Similarly, obese women had greater fat mass than lean women, whether pregnant or non-pregnant. There were no significant differences between baseline estimates of fat mass in pregnant compared with non-pregnant women, both lean and obese. While there was an increase in mean fat mass from baseline to visit 2 in kilograms for lean pregnant women, obese women did not appear to similarly expand their fat mass with increasing gestation. Again, while there was no significant difference in change in fat mass in kg between lean and obese pregnant women, when this was expressed as percentage change from baseline, lean women had a greater expansion of fat mass compared with obese women.

Table 4.2 Longitudinal Changes in Body Composition

	Pregnant			Non-pregnant		
Weight (kg)	Lean	Obese	p^a	Lean	Obese	p^b
Baseline	63.7 ± 1.9 (n = 6)	118.0 ± 3.1 (n = 9)	<0.0001	63.0 ± 3.1 (n = 7)	109.8 ± 3.9 (n = 7)	< 0.0001
Visit 2	71.9 ± 3.1 (n = 4)	124.9 ± 4.3 (n = 5)	< 0.0001			
p ^c	0.02	0.04				
Δ Weight (kg)	8.2 ± 1.8	5.2 ± 1.7	ns			
Δ Weight (%)	13.0 ± 2.9	4.5 ± 1.6	0.03			
Fat Mass (kg)						
Baseline	20.7 ± 1.4	58.1 ± 2.5	< 0.0001	16.5 ± 2.5	54.7 ± 3.4	< 0.0001
Visit 2	25.7 ± 1.2	61.1 ± 3.3	0.02			
p ^d	0.04	ns				
Mean Δ Fat Mass (kg)	5.4 ± 1.6	1.3 ± 2.6	ns			
Mean Δ Fat Mass (%)	30.0 ± 10.8	1.6 ± 2.4	0.004			

Data are expressed as mean ± SEM; ns = not significant.

Details are given for all patients who had satisfactory screening and went on to attend first clamp study, regardless of later exclusion/withdrawal.

a Lean pregnant versus obese pregnant, unpaired Students' t-test.

b Lean non-pregnant versus obese non-pregnant, unpaired Students' t-test.

c Weight, baseline versus visit 2, paired t-test.

d Fat mass, baseline versus visit 2, paired t-test.

4.3.3. Clamped glucose and isotopic enrichments

Plasma glucose was clamped at 4.6mmol/L (figure 4.2A). Mean plasma insulin concentrations of 24.2 ± 0.9 mU/mL were achieved for low dose HEC and 42.6 ± 1.2 mU/mL for high dose HEC (Figure 4.2B). Achieved plasma insulin was slightly greater in obese compared to lean subjects (Figure 4.3) which may reflect reduced insulin clearance in obese subjects. There was an overall effect of BMI when all lean subjects were compared with all obese subjects ($p < 0.001$, 2-way ANOVA) but no between group differences were identified at any specific time points on post-hoc testing. When obese and lean were compared at different time points (ie., non-pregnant, 19 weeks gestation or 36 weeks gestation), there was an effect of BMI at 19 weeks only. Expression of insulin sensitivity as M/I (p162) takes these differences into account by correcting for achieved plasma insulin.

Isotopic enrichment at steady state was achieved for all groups (Figure 4.4). Mean CV for isotopic enrichment at steady state was $5.0 \pm 0.3\%$ (glucose) and $16.3 \pm 1.0\%$ (glycerol).

Figure 4.2 Clamped plasma glucose and insulin

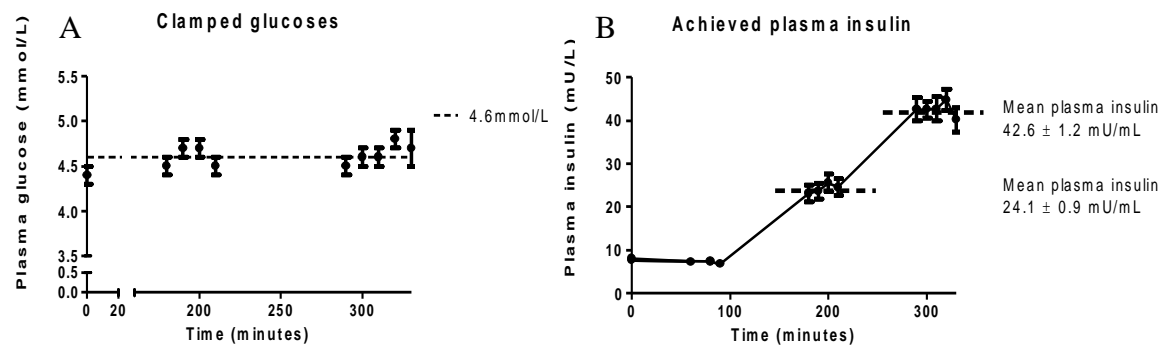


Figure 4.2. (A) Mean plasma glucose throughout the clamp procedure for all subjects. (B) Mean plasma insulin throughout the clamp procedure for all subjects. Data are expressed as mean ± SEM.

Figure 4.3 Achieved plasma insulin in lean compared with obese subjects

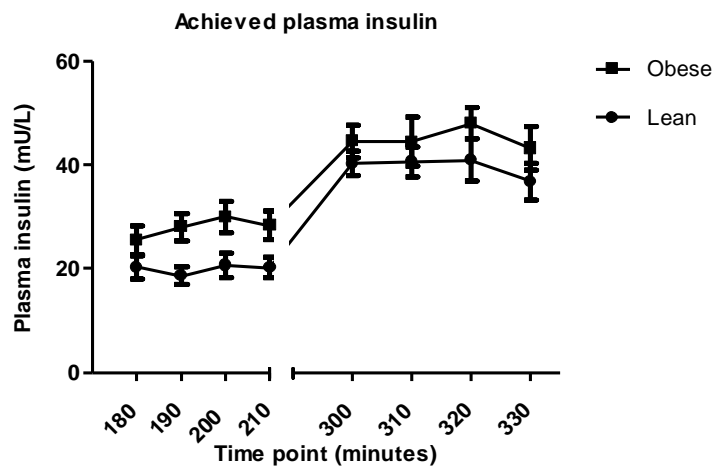


Figure 4.3. Achieved plasma insulin throughout the clamp procedure for all subjects. Data are expressed as mean ± SEM. Overall effect of BMI, $p < 0.001$; no specific between group differences. Data analysed by 2-way ANOVA with Bonferroni post-hoc testing.

Figure 4.4 Plasma isotopic enrichments

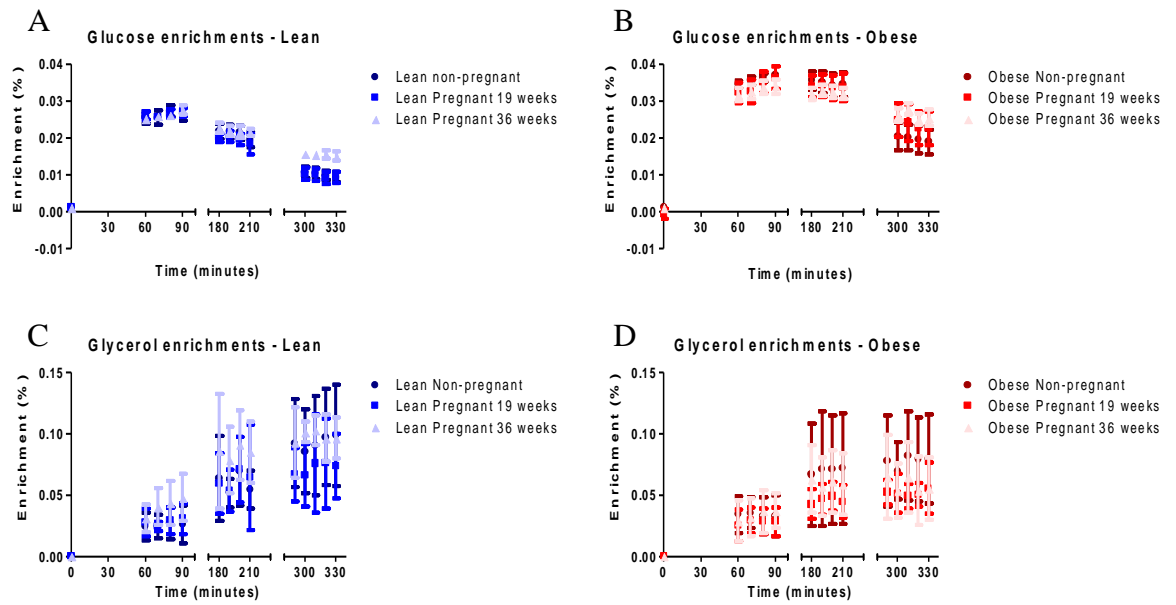


Figure 4.4. d₂-Glucose enrichment in lean (A) and obese (B) subjects and d₅-glycerol enrichment in lean (C) and obese (D) subjects. Data are presented as mean \pm SEM.

4.3.4. Whole body glucose disposal

Whole body glucose disposal (WGD) was calculated in mg glucose/kg total body weight/minute at steady state during high-dose clamp conditions and is an indirect measure of whole body insulin sensitivity (IS), where a greater glucose disposal rate implies greater insulin sensitivity. Insulin sensitivity can also be expressed as M/I, in mg/kg/min/mU/L, an expression of glucose disposal per unit plasma insulin; this also corrects for slight differences in achieved plasma insulin in different groups.

WGD is shown in Figure 4.5 and M/I is shown in Figure 4.6. Obese non-pregnant women had significantly lower IS as measured by WGD compared with lean non-pregnant women as expected. Expressed as M/I, obese non-pregnant women also had lower insulin sensitivity though this was not statistically significant in this cohort.

In lean pregnant women, IS by both indices was maintained or slightly increased at 19 weeks gestation compared with non-pregnant, followed by a significant decrease of approximately 50% by 36 weeks.

In contrast, in obese women, while there was no significant difference in IS at 19 weeks compared with non-pregnant, there was no corresponding decrement in IS between 19 and 36 weeks.

At 19 weeks gestation, obese women had lower IS compared with lean pregnant women of the same gestation; by 36 weeks gestation, there was no significant difference in IS between lean and obese pregnant women.

Figure 4.5 Whole body glucose disposal in lean and obese women

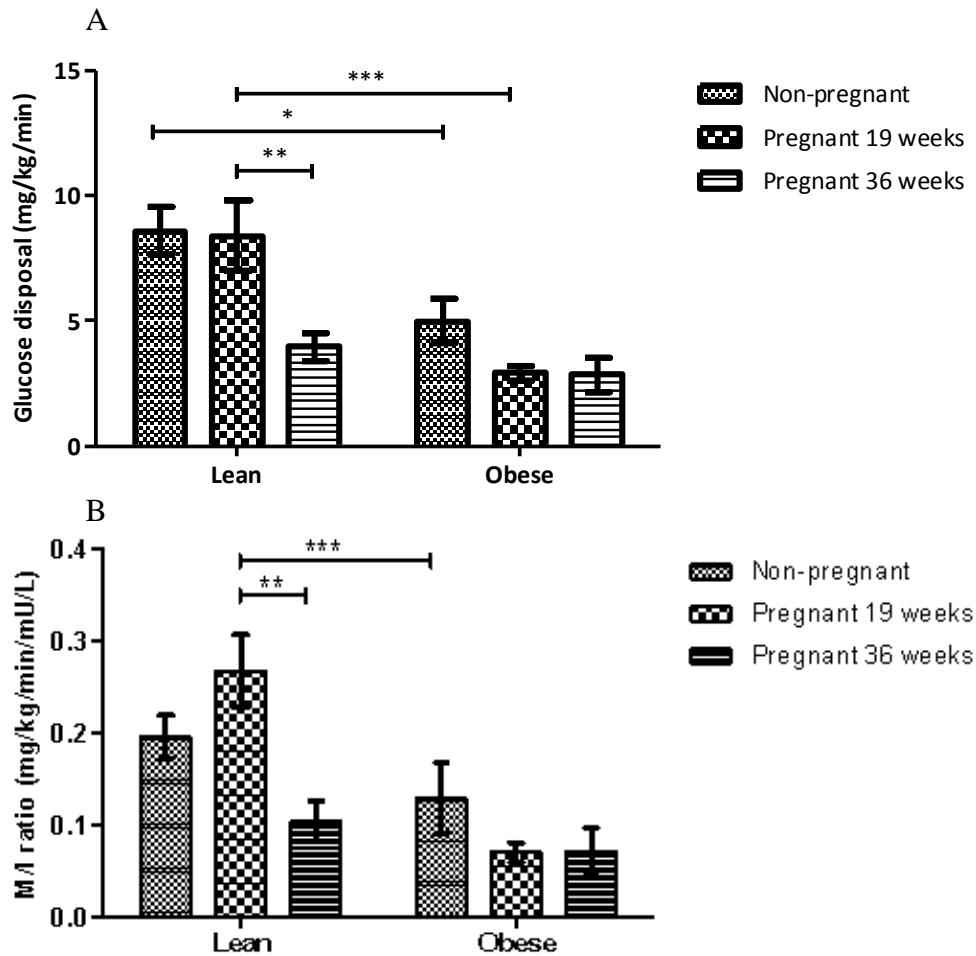


Figure 4.5.(A) Whole body glucose disposal and (B) M/I ratios in lean and obese women

Data are expressed as mean \pm SEM.

Data were analysed using 2-way analysis of variance with Bonferroni post-hoc testing.

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Data are shown for all participants with satisfactory screening who went on to attend Visit 1, regardless of later withdrawal/exclusion. Following withdrawal/exclusion following diagnosis of GDM, group numbers are as follows:

Lean non-pregnant (n=7); lean pregnant 19 weeks (n=6); lean pregnant 36 weeks (n=4).

Obese non-pregnant (n=7); obese pregnant 19 weeks (n=9); obese pregnant 36 weeks (n=5).

4.3.5. Endogenous Glucose Production & Glucose disposal

The rate of disappearance (Rd) of glucose and endogenous glucose production (EGP) are shown in Figure 4.6 (expressed per kg total body weight).

At baseline, prior to insulin administration, Rd glucose is equivalent to EGP as they are in steady state. The time point before insulin administration represents fasting conditions. Fasting Rd glucose was significantly greater in lean compared to obese women in pregnancy at both gestations and in non-pregnant women (4.6A).

Following low dose insulin infusion, Rd glucose was increased compared to baseline in all groups (4.6B). Rd glucose was greater in lean compared to obese non-pregnant women and in pregnant women at 19 but not 36 weeks. Following high dose insulin infusion, Rd glucose was further increased, more so in lean compared to obese women (4.6C). The increase in Rd glucose in response to high dose insulin was most pronounced in lean women at 19 weeks, with a smaller response in this group at 36 weeks gestation. No such difference in response was observed in obese women.

Endogenous glucose production was reduced in all groups compared to fasting conditions pre-insulin (4.6D). In lean women, there was a small but statistically significant increase in EGP at 19 weeks gestation compared to non-pregnant women. At this gestation, EGP was greater in lean compared to obese women but there were no statistically significant differences between lean and obese non-pregnant women by 36 weeks gestation.

Rd glucose and EGP can also be expressed per kg fat-free mass, representing substrate uptake by non-adipose tissue (Figure 4.7). Under fasting conditions, there were no differences in Rd glucose per kg between lean and obese non-pregnant subjects or in pregnant subjects at 19 weeks gestation. However, by 36 weeks gestation, there was significantly greater glucose uptake in both lean and obese pregnant women, although no significant differences between lean or obese women (Figure 4.7A).

Following low dose insulin, glucose uptake was stimulated in both lean and obese women, except at 36 weeks gestation where this did not appear to occur (Figure 4.7B).

Following high dose insulin infusion, glucose uptake was increased in lean and obese non-pregnant women, and in lean but not obese women in early pregnancy (Figure 4.7C). By 36 weeks, there was little further stimulation of glucose uptake in both lean and obese women. In lean pregnant women, stimulation of glucose uptake was reduced at 36 compared with 19 weeks.

Endogenous glucose production under fasting conditions is equal to Rd glucose (Figure 4.7A) and was almost completely suppressed by low dose insulin (Figure 4.7E). At high dose insulin, there is no reliable measure of EGP due to artefact from large volumes of non-labelled glucose infused to maintain euglycaemia.

Figure 4.6.

Rd glucose and endogenous glucose production per kg total bodyweight

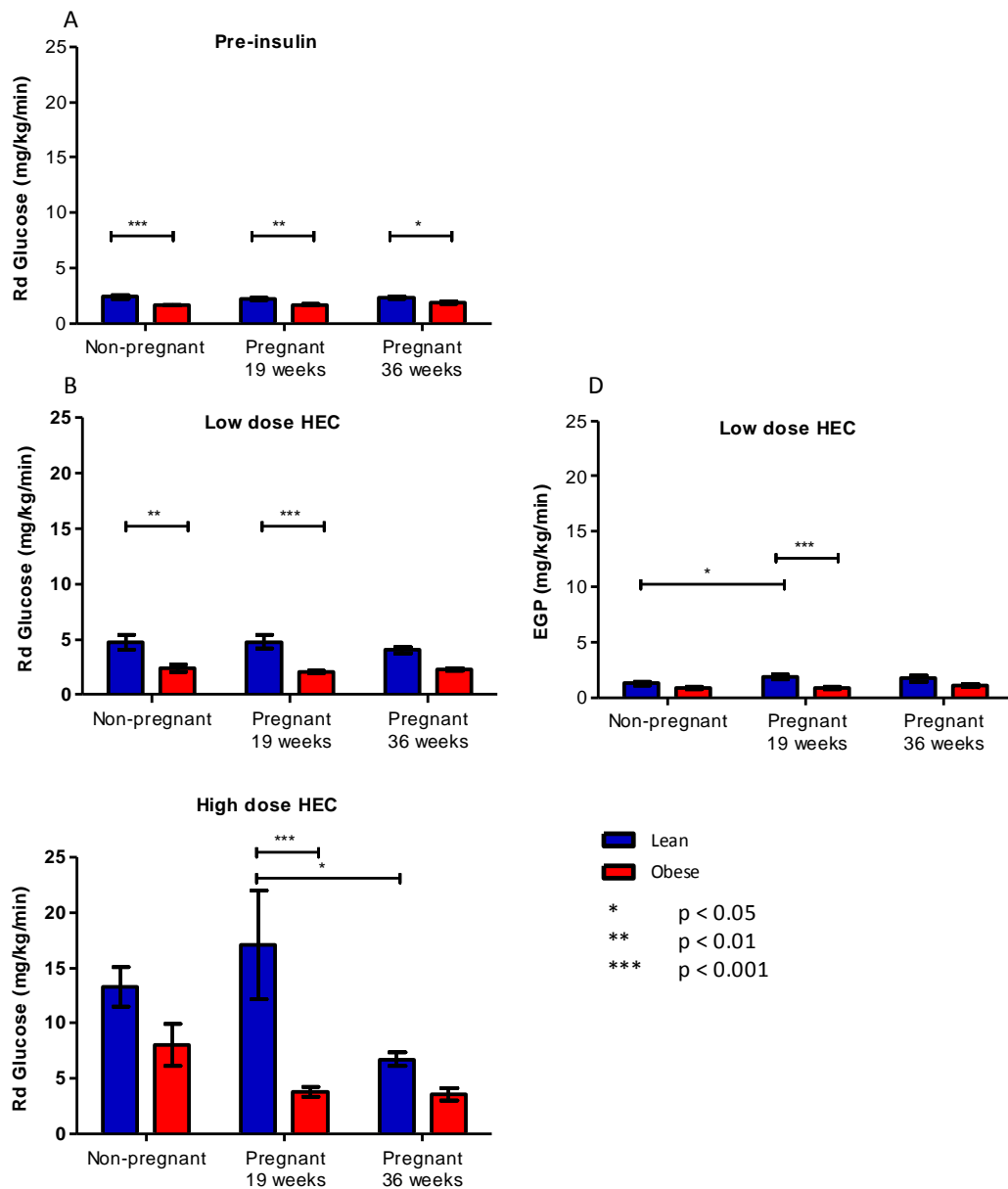


Figure 4.6. (A, B and C) Rd glucose per kg total body weight under fasting conditions (A) and following low dose (B) and high dose (C) insulin infusion. (D) Endogenous glucose production (EGP), following low dose insulin infusion.

Data are presented as mean \pm SEM. * p < 0.05 ** p < 0.01 *** p < 0.001

Data were analysed using 2-way analysis of variance with Bonferroni post-hoc testing.

Data are shown for all participants with satisfactory screening who went on to attend Visit 1, regardless of later withdrawal/exclusion. Following withdrawal/exclusion following diagnosis of GDM, group numbers are as follows:

Lean non-pregnant (n=7); lean pregnant 19 weeks (n=6); lean pregnant 36 weeks (n=4).

Obese non-pregnant (n=7); obese pregnant 19 weeks (n=9); obese pregnant 36 weeks (n=5).

Figure 4.7.

Rd glucose and endogenous glucose production per kg fat-free mass

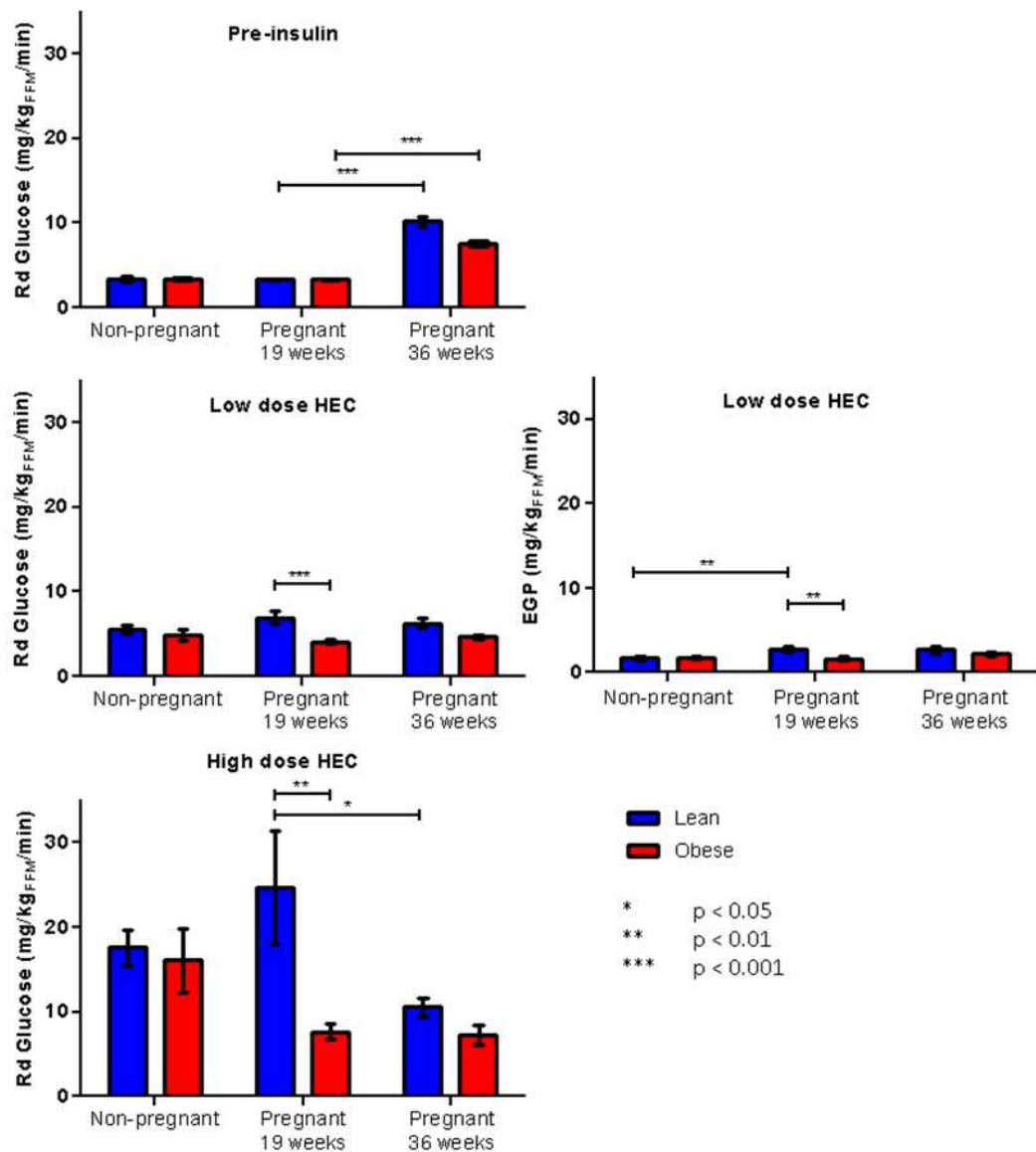


Figure 4.7. (A, B and C) Rd glucose per kg fat-free mass under fasting conditions (A) and following low dose (B) and high dose (C) insulin infusion. (D) Endogenous glucose production (EGP), following low dose insulin infusion.

Data are presented as mean \pm SEM. * p < 0.05 ** p < 0.01 *** p < 0.001

Data were analysed using 2-way analysis of variance with Bonferroni post-hoc testing.

Data are shown for all participants with satisfactory screening who went on to attend Visit 1, regardless of later withdrawal/exclusion. Following withdrawal/exclusion following diagnosis of GDM, group numbers are as follows:

Lean non-pregnant (n=7); lean pregnant 19 weeks (n=6); lean pregnant 36 weeks (n=4).

Obese non-pregnant (n=7); obese pregnant 19 weeks (n=9); obese pregnant 36 weeks (n=5).

4.3.6. Lipolysis

Rate of appearance (Ra) of glycerol per kg fat mass is shown in Figure 4.8 (A, B & C). In lean women, baseline pre-insulin rate of lipolysis per kg fat mass was greatest in non-pregnant women, reducing in pregnancy and lowest at 36 weeks (4.8A). In obese women, there was no such response to pregnancy. In non-pregnant women, obese women had a much lower rate of lipolysis when fasting compared with lean women. In pregnancy, there was no difference in glycerol production between lean and obese women.

Low dose insulin infusion resulted in suppression of Ra glycerol in all groups (4.8B). In non-pregnant women, Ra glycerol remained lower in obese compared with lean women; there was no difference between pregnant and non-pregnant groups.

High dose insulin infusion resulted in little further suppression of Ra glycerol compared with low dose insulin (4.8C).

At isotopic steady state, the rate of appearance (Ra) glycerol is equal to the rate of disappearance (Rd) glycerol. Since adipose tissue is the only source of glycerol production, Ra glycerol per kg fat mass is indicative of the rate of lipolysis. Rd glycerol is then expressed per kg fat-free mass (Figure 4.8D, E & F) and is indicative of substrate utilisation. There is no re-uptake of glycerol by adipose tissue, therefore fat-free mass alone is responsible for glycerol uptake.

Under fasting conditions, utilisation of glycerol as a substrate is increased in both lean and obese women in response to pregnancy (4.8D) and is slightly greater in obese compared to lean women although this is not statistically significant. Glycerol utilisation is suppressed in response to insulin infusion (4.8E & F) with greater suppression in lean compared to obese women.

The measurements of glycerol enrichment have a high variance, this is due to the very low levels of glycerol in these samples, resulting in difficulties with the GC-MS assay; this may lead to potential problems with reliability of these data, particularly between group analyses in a small cohort. However, there is clear suppression of glycerol production by insulin infusion in all subjects as expected.

Figure 4.8. Whole body glycerol production

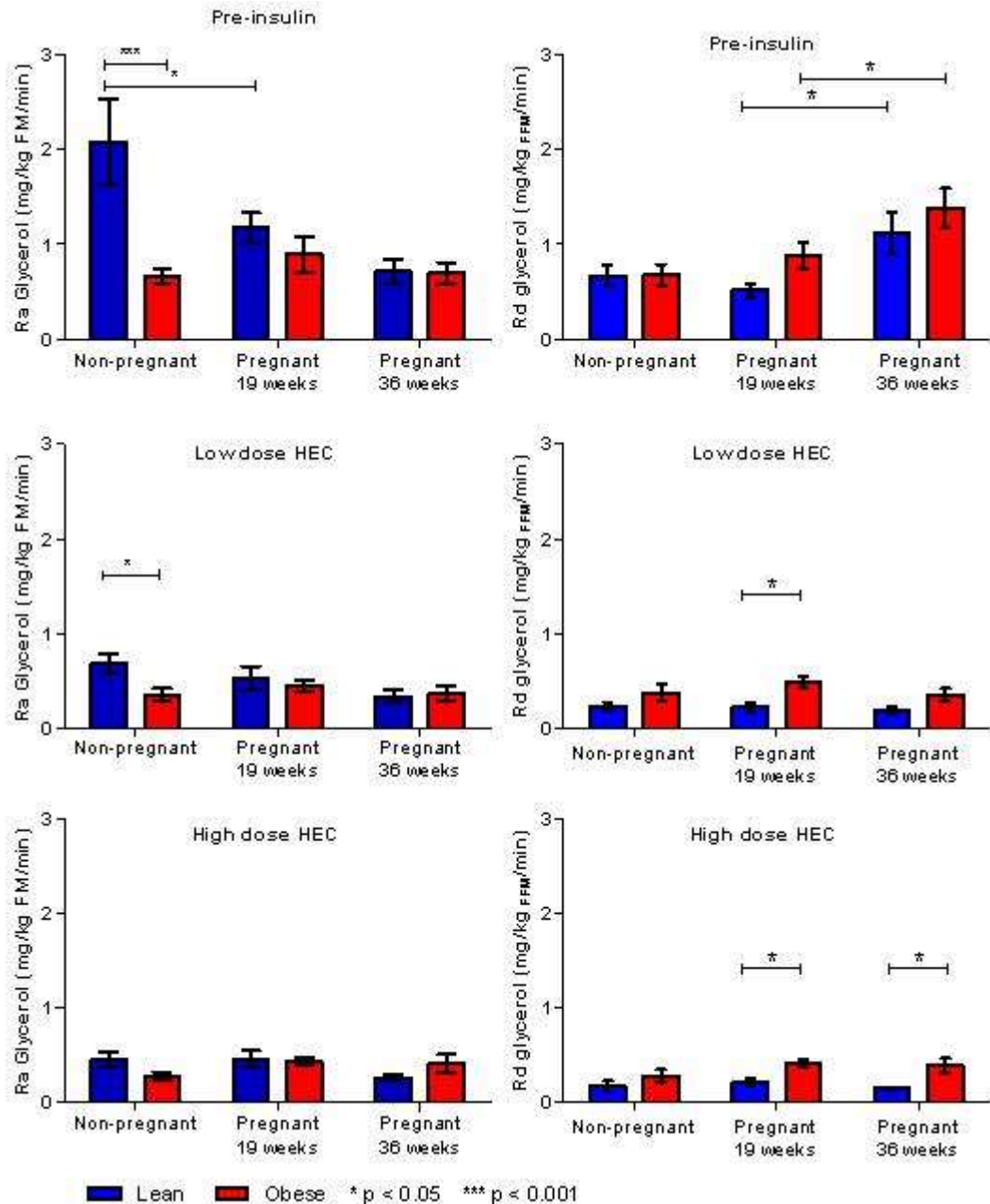


Figure 4.8. Ra glycerol per kg fat mass under fasting conditions (A) and following low dose (B) and high dose (C) insulin infusion. Rd glycerol per kg fat-free mass under fasting conditions (D) and following low dose (E) and high dose (F) insulin infusion. Data are presented as mean \pm SEM. Data were analysed using 2-way analysis of variance with Bonferroni post-hoc testing. Data are shown for all participants with satisfactory screening who went on to attend Visit 1, regardless of later withdrawal/exclusion. Following withdrawal/exclusion following diagnosis of GDM, group numbers are as follows: Lean non-pregnant (n=7); lean pregnant 19 weeks (n=6); lean pregnant 36 weeks (n=4). Obese non-pregnant (n=7); obese pregnant 19 weeks (n=9); obese pregnant 36 weeks (n=5).

4.3.7. Circulating lipids & insulin

Total cholesterol and triglycerides are shown in Figure 4.9. There was no difference in total cholesterol between lean and obese individuals, either non-pregnant, at 19 weeks gestation or at 36 weeks (Figure 4.9A). However, there was a significant increase in total cholesterol in lean but not obese women from 19 to 36 weeks ($p < 0.05$). At 36 weeks, triglycerides were significantly greater compared with the respective non-pregnant group in both lean and obese women (Figure 4.9B). In lean pregnant women, this increase occurred late, with a significant increase between 19 and 36 weeks gestation ($p < 0.05$); whereas in obese women, triglycerides were already elevated and there was no further significant increase in triglyceride by 36 weeks gestation.

Serum NEFA levels are shown in Figure 4.10. In lean women, there were no significant differences in mean serum NEFA between non-pregnant and pregnant women. In obese women, there was a significant increase in serum NEFA from non-pregnant to 19 weeks, with no further increase by 36 weeks gestation. At 19 weeks gestation, obese women had significantly greater serum NEFA than lean women ($p < 0.05$); by 36 weeks, there was no significant difference between lean and obese women.

Serum insulin concentrations are shown in Figure 4.11. In lean women, there were no significant differences in mean fasting serum insulin between pregnant and non-pregnant women. In obese women, mean serum insulin increased from non-pregnant to 19 weeks, but there was no further increase during the remainder of pregnancy.

Obese women had greater serum insulin compared to lean women throughout pregnancy but not in the non-pregnant state.

Figure 4.9 Circulating lipids

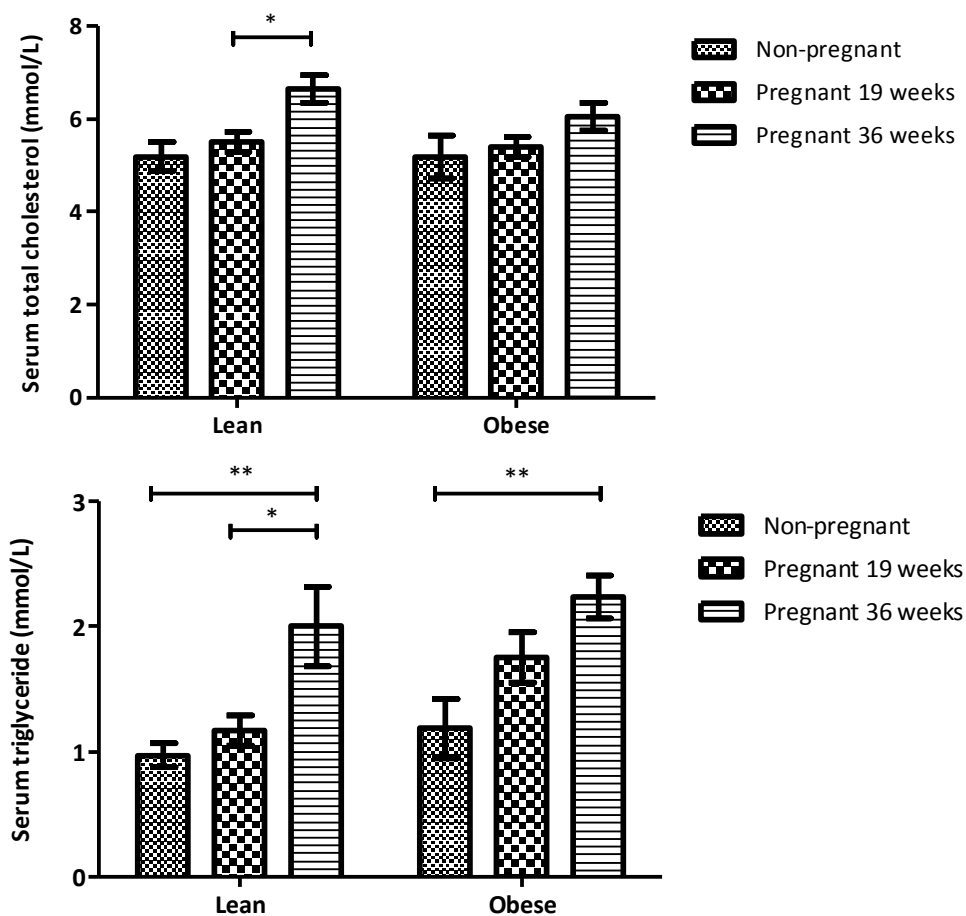


Figure 4.9 Fasting serum total cholesterol and triglyceride

Data are expressed as mean \pm SEM. * $p < 0.05$ ** $p < 0.01$

- A. Serum total cholesterol.
- B. Serum triglyceride.

Data were analysed using 2-way analysis of variance with Bonferroni post-hoc testing.

Data are shown for all participants with satisfactory screening who went on to attend Visit 1, regardless of later withdrawal/exclusion. Following withdrawal/exclusion following diagnosis of GDM, group numbers are as follows:

Lean non-pregnant (n=7); lean pregnant 19 weeks (n=6); lean pregnant 36 weeks (n=4).

Obese non-pregnant (n=7); obese pregnant 19 weeks (n=9); obese pregnant 36 weeks (n=5).

Figure 4.10 Serum NEFA

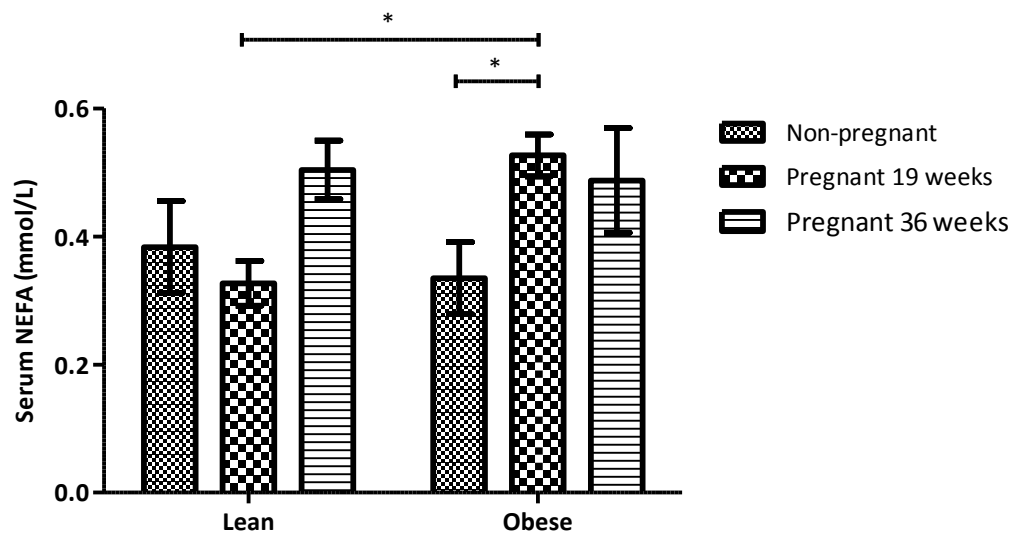


Figure 4.10 Serum NEFA

Data are expressed as mean \pm SEM. * $p < 0.05$

Data were analysed using 2-way analysis of variance with Bonferroni post-hoc testing.

Data are shown for all participants with satisfactory screening who went on to attend Visit 1, regardless of later withdrawal/exclusion. Following withdrawal/exclusion following diagnosis of GDM, group numbers are as follows:

Lean non-pregnant (n=7); lean pregnant 19 weeks (n=6); lean pregnant 36 weeks (n=4).

Obese non-pregnant (n=7); obese pregnant 19 weeks (n=9); obese pregnant 36 weeks (n=5).

Figure 4.11 Fasting serum insulin

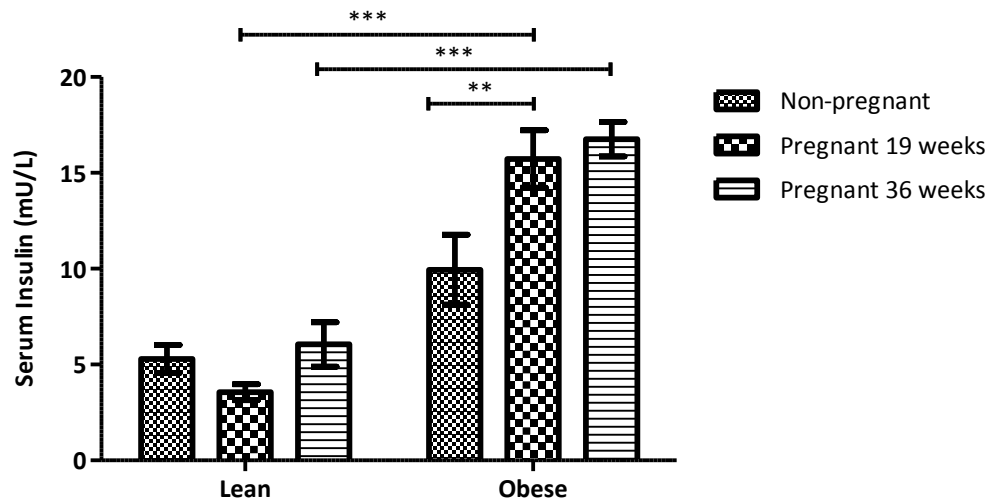


Figure 4.11 Serum insulin

Data are expressed as mean \pm SEM. ** $p < 0.01$ *** $p < 0.001$

Data were analysed using 2-way analysis of variance with Bonferroni post-hoc testing.

Data are shown for all participants with satisfactory screening who went on to attend Visit 1, regardless of later withdrawal/exclusion. Following withdrawal/exclusion following diagnosis of GDM, group numbers are as follows:

Lean non-pregnant (n=7); lean pregnant 19 weeks (n=6); lean pregnant 36 weeks (n=4).

Obese non-pregnant (n=7); obese pregnant 19 weeks (n=9); obese pregnant 36 weeks (n=5).

4.3.8. Adipokines

Plasma leptin and mRNA transcript levels of leptin in abdominal subcutaneous adipose tissue are shown in Figure 4.12. Plasma levels of leptin were greater in both non-pregnant and pregnant obese compared with matched lean subjects with an overall effect of BMI ($p < 0.0001$) (Figure 4.12A). Transcript levels of leptin in abdominal subcutaneous adipose tissue were significantly greater in obese compared with lean non-pregnant subjects (Figure 4.12B) ($p < 0.01$). In lean subjects, transcript levels of leptin were elevated in pregnant compared with non-pregnant subjects, although this was not statistically significant. In obese subjects, transcript levels of leptin were reduced in pregnant compared with non-pregnant subjects.

Plasma total adiponectin and transcript levels of adiponectin in abdominal subcutaneous adipose tissue are shown in Figure 4.13. In lean women, total adiponectin (Figure 4.13A) was greater in pregnant compared with non-pregnant women (at 19 weeks, $p < 0.05$); however, there was no further change between 19 and 36 weeks. In obese women, there was no difference between pregnant and non-pregnant subjects. At 19 weeks, obese subjects had significantly lower total plasma adiponectin compared with lean pregnant subjects ($p < 0.01$); at 36 weeks, there was no significant difference between lean and obese women. Transcript levels of adiponectin (Figure 4.13B) were lower in obese compared with lean non-pregnant and pregnant subjects, with an overall effect of BMI, ($p < 0.01$). There were no statistically significant specific between-group differences.

Figure 4.12 Leptin

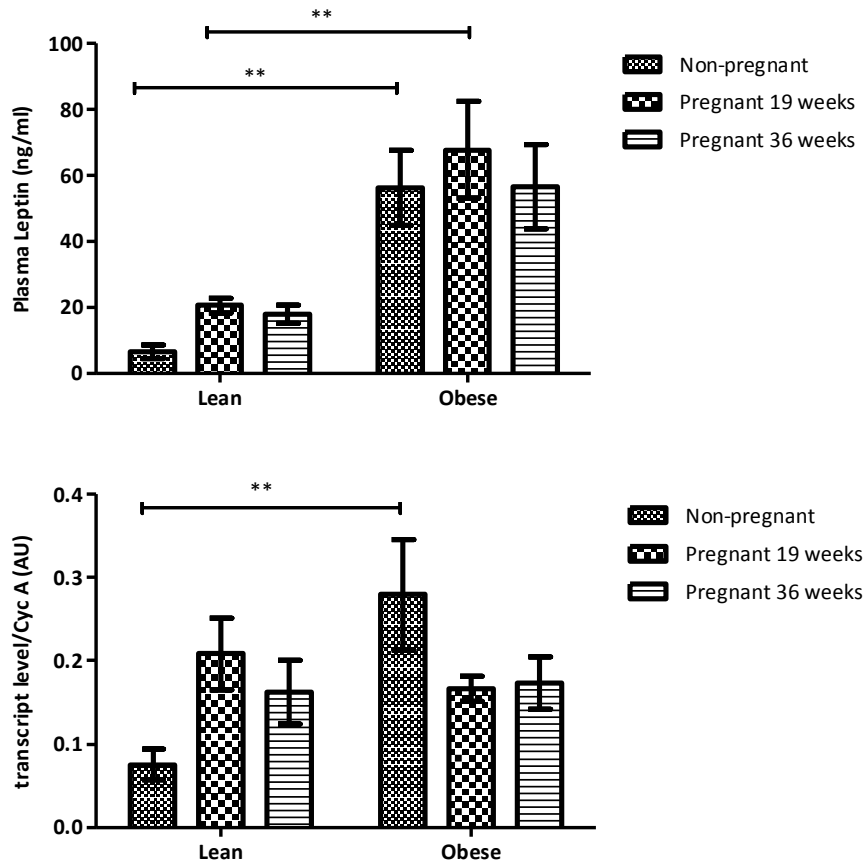


Figure 4.12 Plasma Leptin & transcript levels of leptin in subcutaneous abdominal adipose tissue

Leptin was measured in plasma by ELISA. Transcript levels of leptin were measured in adipose tissue by RT-PCR and normalised to transcript levels of cyclophilin A.

A. Plasma leptin.

B. Transcript levels of leptin in subcutaneous abdominal adipose tissue.

Data are expressed as mean \pm SEM. ** $p < 0.01$. Data were analysed using 2-way analysis of variance with Bonferroni post-hoc testing.

Data are shown for all participants with satisfactory screening who went on to attend Visit 1, regardless of later withdrawal/exclusion. Following withdrawal/exclusion following diagnosis of GDM, group numbers are as follows:

Lean non-pregnant (n=7); lean pregnant 19 weeks (n=6); lean pregnant 36 weeks (n=5, plasma; n=4, transcript).

Obese non-pregnant (n=7); obese pregnant 19 weeks (n=9); obese pregnant 36 weeks (n=4, plasma; n=5, transcript).

Figure 4.13 Adiponectin

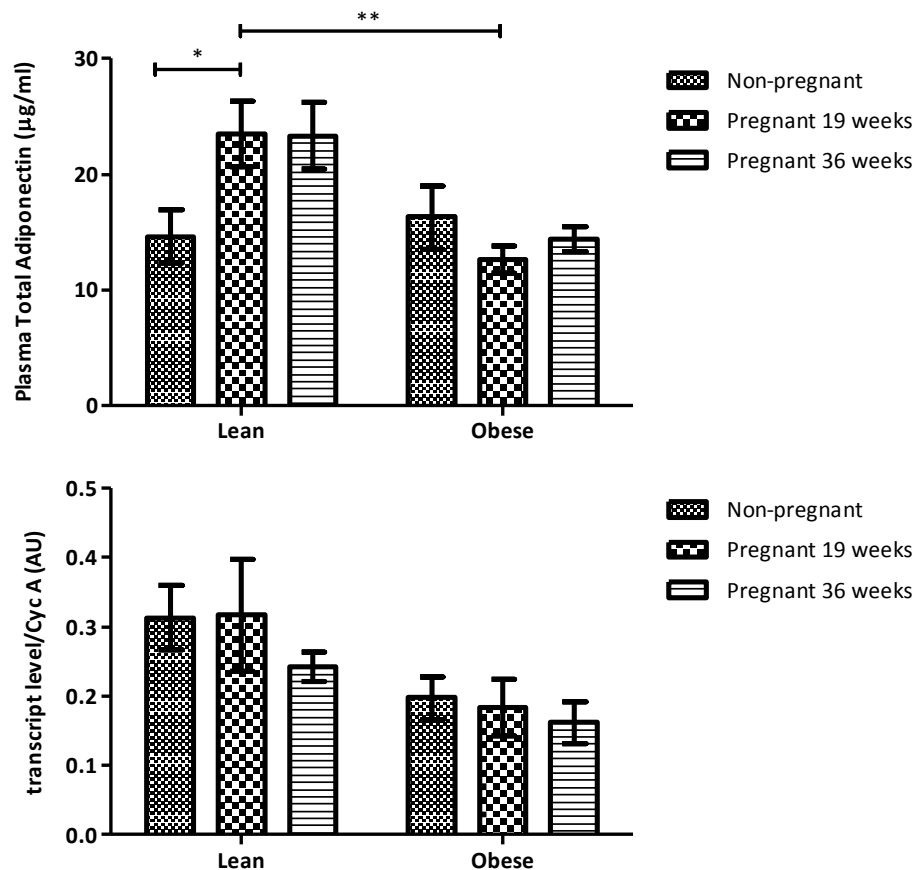


Figure 4.13 Plasma Total Adiponectin & transcript levels of adiponectin in subcutaneous abdominal adipose tissue

Adiponectin was measured in plasma by ELISA. Transcript levels of adiponectin were measured in adipose tissue by RT-PCR and normalised to transcript levels of cyclophilin A.

A. Plasma total adiponectin.

B. Transcript levels of adiponectin in subcutaneous abdominal adipose tissue.

Data are expressed as mean \pm SEM. * $p < 0.05$ ** $p < 0.01$. Data were analysed using 2-way analysis of variance with Bonferroni post-hoc testing.

Data are shown for all participants with satisfactory screening who went on to attend Visit 1, regardless of later withdrawal/exclusion. Following withdrawal/exclusion following diagnosis of GDM, group numbers are as follows:

Lean non-pregnant (n=7); lean pregnant 19 weeks (n=6); lean pregnant 36 weeks (n=5, plasma; n=4, transcript).

Obese non-pregnant (n=7); obese pregnant 19 weeks (n=9); obese pregnant 36 weeks (n=4, plasma; n=5, transcript).

4.3.9. Circulating plasma cytokines

Plasma IL-6 and IL-1Ra were quantified in plasma (Figure 4.14). In lean women, plasma IL-6 levels were greater in pregnant compared with non-pregnant subjects (Figure 4.14A). In obese women, there was no difference between non-pregnant subjects and pregnant women at 19 weeks gestation; at 36 weeks, there was an increase in plasma IL-6 though this was not statistically significant.

In lean women, plasma IL-1Ra was greater in pregnant compared with non-pregnant women, and increased with gestation although this was not statistically significant (Figure 4.14B). In obese women, plasma IL-1Ra was increased at 19 weeks compared with non-pregnant and then decreased at 36 weeks.

Figure 4.14 Circulating plasma cytokines

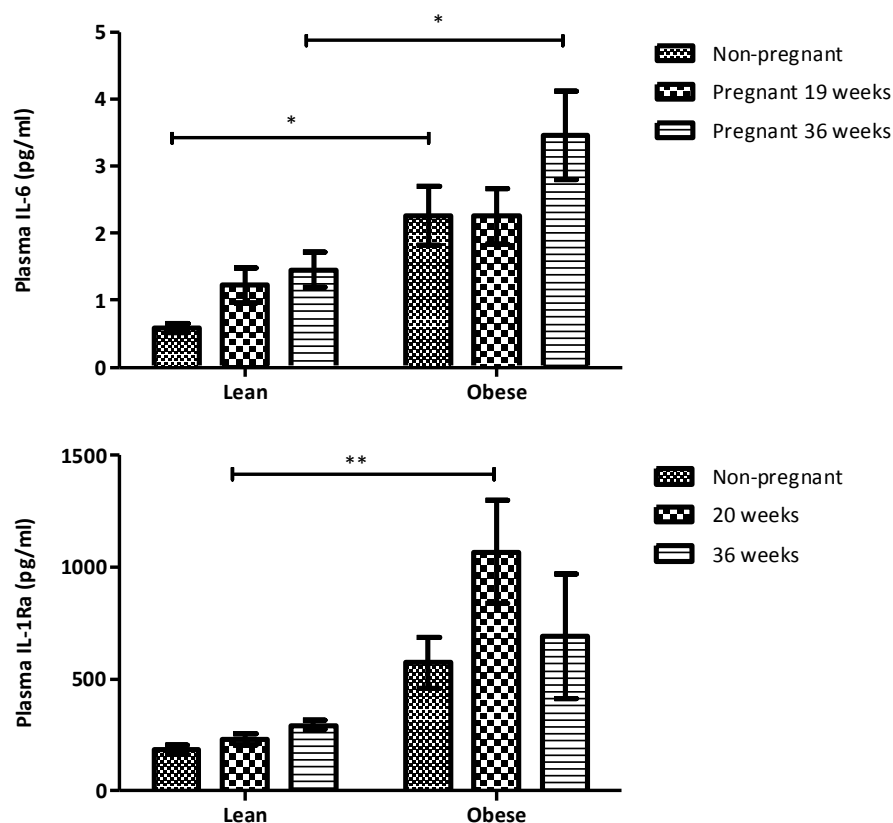


Figure 4.14 Plasma IL-6 (A) and IL-1Ra (B), measured by ELISA

Data are expressed as mean \pm SEM. * $p < 0.05$ ** $p < 0.01$

Data were analysed using 2-way analysis of variance with Bonferroni post-hoc testing.

Data are shown for all participants with satisfactory screening who went on to attend Visit 1, regardless of later withdrawal/exclusion. Following withdrawal/exclusion following diagnosis of GDM, group numbers are as follows:

Lean non-pregnant (n=7); lean pregnant 19 weeks (n=6); lean pregnant 36 weeks (n=5).

Obese non-pregnant (n=7); obese pregnant 19 weeks (n=9); obese pregnant 36 weeks (n=4).

4.3.10. Pro-inflammatory transcripts in abdominal subcutaneous adipose tissue

Transcript levels of IL-6, MCP-1 and TNF α were quantified in subcutaneous adipose tissue using real time RT-PCR (Taqman) (Figure 4.15).

Transcript levels of IL-6 (Figure 4.15A) were broadly greater in obese compared with lean women in both pregnant and non-pregnant groups; in both lean and obese women, there appeared to be an increase in IL-6 transcript levels in response to pregnancy. There was an overall effect of BMI ($p = 0.05$) but there were no significant between-group differences between lean or obese women at any specific time points.

In lean women, transcript levels of MCP1 (Figure 4.15B) increased in pregnant compared with non-pregnant women and there was a further increase from 19 to 36 weeks, although this was not statistically significant in this cohort. In obese women, there was a small but non-significant increase in transcript levels of MCP1 in pregnant women at 19 weeks gestation compared with non-pregnant obese women but no further increase in pregnancy. In non-pregnant women, transcript levels of MCP1 were greater in obese compared to lean women, but in pregnancy, there were no significant differences between lean and obese women.

TNF α transcript levels (Figure 4.15C) were significantly increased in lean pregnant women at 19 weeks gestation compared with lean non-pregnant women ($p = 0.05$), with no further change during pregnancy. There was no effect of pregnancy in obese women. Transcript levels of TNF α were greater in obese compared with lean non-pregnant women, although this was not statistically significant in this cohort; there was no significant difference between lean and obese pregnant women.

Figure 4.15 Pro-inflammatory transcripts in abdominal subcutaneous adipose tissue

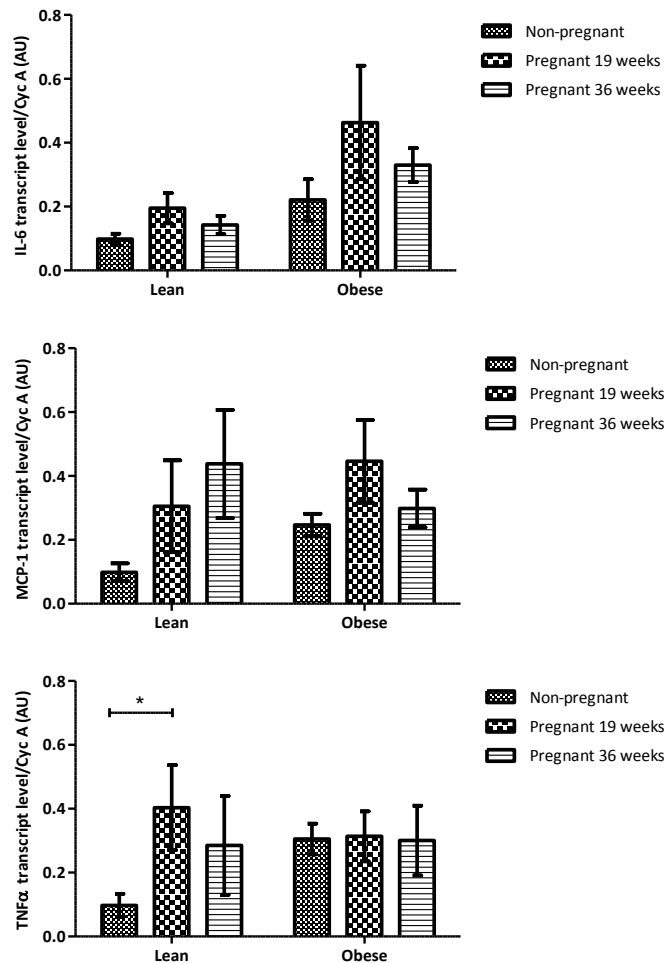


Figure 4.15 Transcript of pro-inflammatory cytokines
Transcript levels of IL-6 (A), MCP-1 (B) and TNFα (C) were quantified by real time RT-PCR and expressed relative to transcript levels of cyclophilin A.

Data are expressed as mean \pm SEM. * $p < 0.05$

Data were analysed using 2-way analysis of variance with Bonferroni post-hoc testing.

Data are shown for all participants with satisfactory screening who went on to attend Visit 1, regardless of later withdrawal/exclusion. Following withdrawal/exclusion following diagnosis of GDM, group numbers are as follows:

Lean non-pregnant (n=7); lean pregnant 19 weeks (n=6); lean pregnant 36 weeks (n=5).

Obese non-pregnant (n=7); obese pregnant 19 weeks (n=9); obese pregnant 36 weeks (n=4).

4.3.11. Metabolic transcripts in abdominal subcutaneous adipose tissue

In our group, microarray analysis was carried out to identify potential candidate genes whose expression was altered in response to the combination of pregnancy and obesity, using adipose tissue from a murine model of diet induced obesity (Pedroni, Morton, *et al.* Unpub. 2012). They found that a selection of genes which regulate lipid synthesis and storage were specifically upregulated in obese pregnant animals. To validate these genes in a human model, transcript levels of 6 of these genes were assayed in adipose biopsies from subjects undergoing clamp procedures. Transcript levels of stearoyl-CoA desaturase-1 (SCD1), malic enzyme-1 (ME1), retinol binding protein-4 (RBP4), fatty acid synthase (FASN), diglycerol acyl transferase-2 (DGAT2) and insulin receptor substrate-1 (IRS1) were quantified in subcutaneous adipose tissue using quantitative real time RT-PCR (Taqman) (Figure 4.16).

In obese women, transcript levels of SCD1 (Figure 4.16A) were decreased in early pregnancy compared with non-pregnant ($p < 0.05$). No such decrease was seen at 19 weeks in lean women compared with non-pregnant; however, by 36 weeks, SCD1 transcript levels were decreased by approximately 60% to levels comparable with obese subjects, though this was not statistically significant in this cohort.

In obese women, ME1 transcript levels (Figure 4.16B) were decreased compared with lean women with an overall effect of BMI ($p = 0.05$). In both lean and obese women, there was a decrease in ME1 transcript levels associated with pregnancy.

There was no difference in transcript levels of RBP4 (Figure 4.16C) in lean or obese women, nor was there a difference between pregnant and non-pregnant groups.

Transcript levels of FASN (Figure 4.16D) were decreased in obese compared to lean women, in both pregnant and non-pregnant groups, with an overall effect of BMI ($p < 0.0001$). There was no specific effect of pregnancy.

Transcript levels of DGAT2 (Figure 4.16E) were decreased in obese compared with lean women at each time point and in pregnant women compared with non-pregnant, though this was not statistically significant in this cohort. There was no difference between 19 and 36 weeks gestation in either lean or obese women.

Transcript levels of IRS1 (Figure 4.16F) were decreased in obese compared with lean women in both pregnant and non-pregnant groups with an overall effect of BMI ($p = 0.02$). In lean women, IRS1 transcript levels were increased at 19 weeks compared with non-pregnant but then significantly decreased by 36 weeks ($p < 0.01$); there was no change in IRS1 levels in response to pregnancy in obese women. IRS1 transcript levels were significantly decreased in obese compared to lean pregnant women at 19 weeks gestation ($p < 0.01$); by 36 weeks there was no significant difference between lean and obese women.

Figure 4.16 Metabolic transcripts in abdominal subcutaneous adipose tissue

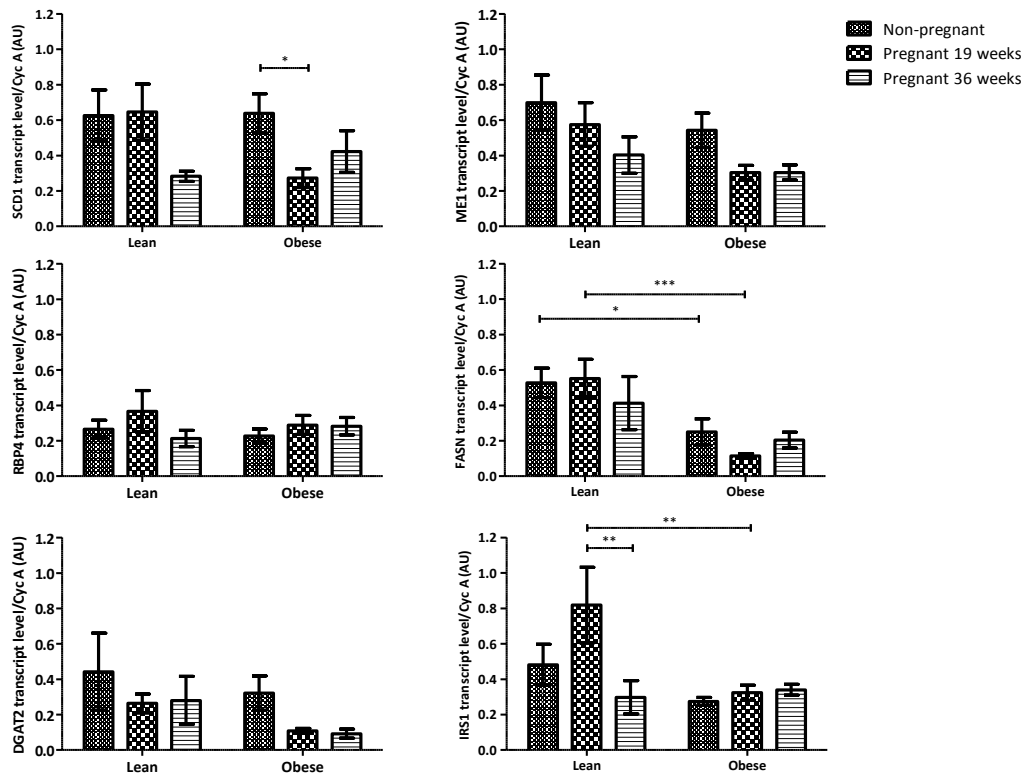


Figure 4.16 Transcript levels of key genes involved in fatty acid regulation

Transcript levels of SCD1 (A), ME1 (B), RBP4 (C), FAS (D), DGAT2 (E) and IRS1 (F) were quantified by real time RT-PCR and expressed relative to transcript levels of cyclophilin A.

Data are expressed as mean \pm SEM; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

Data were analysed using 2-way analysis of variance with Bonferroni post-hoc testing.

Data are shown for all participants with satisfactory screening who went on to attend Visit 1, regardless of later withdrawal/exclusion. Following withdrawal/exclusion following diagnosis of GDM, group numbers are as follows:

Lean non-pregnant (n=7); lean pregnant 19 weeks (n=6); lean pregnant 36 weeks (n=5).

Obese non-pregnant (n=7); obese pregnant 19 weeks (n=9); obese pregnant 36 weeks (n=4).

4.4. Discussion

In the previous chapter, we showed that obese women were insulin resistant throughout pregnancy compare with lean women and that although many features consistent with obesity such as excess circulating inflammatory markers were present, there were some features of the adipose tissue at term in which lean and obese women did not appear to be as divergent as expected. In this chapter, we have shown that in early pregnancy many of these differences persist, but during pregnancy lean and obese women follow a convergent path with advancing gestation, such that by the third trimester, there are many similar metabolic characteristics between women of greatly differing BMI. Indeed, obese women appear resistant to the metabolic changes which occur in lean women during pregnancy. This refutes our initial hypothesis that the combination of obesity and pregnancy would lead to an exaggerated metabolic profile.

In healthy pregnancies, there is progressive development of insulin resistance, maximal in the third trimester and immediate post-partum period (Spellacy, Goetz, *et al.* 1965; Catalano, Tyzbir, *et al.*, 1993). A number of factors have been shown to be important in the development of pregnancy-related IR, including TNF α (Kirwan, Haugel-De Mouzon, *et al.* 2002) and adiponectin (Catalano, Hoegh, *et al.* 2006). One of the main associations of obesity outwith pregnancy is systemic resistance to insulin. We therefore expected to find an additive effect where obesity and pregnancy co-exist, with this effect greatest in the third trimester.

As expected, in non-pregnant women, obese subjects had greatly reduced whole-body insulin sensitivity as assessed by insulin-stimulated whole body glucose disposal compared with lean women (Figures 4.5 and 4.6). However, in contrast to the original hypothesis, in healthy non diabetic obese women,

pregnancy did not appear to exert as great an effect on maternal insulin sensitivity as it did in lean women, and by the third trimester, there were few differences between lean and obese pregnant women. Achieved plasma insulin during the clamp was slightly greater in obese women, consistent with reduced insulin clearance in obese subjects (Mora, Scarfone, *et al.* 2003). However, when insulin sensitivity is expressed as M/I, ie, glucose disposal per unit insulin, a similar pattern of IS is observed, with a large decrement in IS in lean women in pregnancy and relatively little effect of pregnancy in obese women.

Consistent with previous published studies, there was a decrement in insulin sensitivity in lean women of approximately 50-60% between 19 and 36 weeks gestation. No such decrement was observed in obese women. Previous studies have also shown that in overweight and class I obese (BMI > 30kg/m²) women, there is a reduction in the magnitude of change in insulin sensitivity compared with lean women, although some degree of response to pregnancy is maintained (Catalano & Ehrenberg, 2006), as well as development of a greater degree of insulin resistance in obese women who have developed gestational diabetes (Catalano, Huston, *et al.* 1999). This data extends previous work to women of even greater pre-pregnancy BMI and importantly, excludes those with gestational diabetes. Women who develop gestational diabetes are not necessarily those in whom the most severe insulin resistance is found, but those women who are unable to sustain a compensatory hyperinsulinaemia. Data was included in analysis for those women who subsequently developed gestational diabetes (3 x obese, 1 x lean) as these women had normal glucose tolerance as defined by an oral glucose tolerance test at this time of study although they were subsequently excluded from participation in the 2nd study visit. There did not appear to be

any differences in whole body glucose disposal between these women and the rest of their cohorts at 19 weeks gestation, although with such limited numbers, sub-group analysis is not practical. However, it is possible that their inclusion may affect the heterogeneity of the pregnant groups. Following diagnosis of GDM, these women were excluded from further study for two reasons. Firstly women with GDM handle glucose load differently and respond differently to clamp conditions and secondly, women with GDM are managed differently in pregnancy compared to healthy women in terms of nutritional advice and pharmacological interventions including exogenous insulin and metformin.

A further consideration in this cohort is the discrepant ages of the different cohorts (Table 4.1). Increased age is associated with insulin resistance but this is often linked to age-associated accumulation of visceral fat (Kohrt, Kirwan, *et al.* 1993; Gabriely, Ma, *et al.* 2002). While there were statistically significant differences in the ages of the four groups, these differences are not likely to be clinically relevant, particularly as the groups are otherwise well matched in body mass and fat mass. Social class was also not a specific inclusion or exclusion criteria in this cohort; although participants were of social class IIIa and above on the basis of occupation, DEPCAT scoring was not performed; social deprivation is associated with overall poorer health, and risk of diabetes (Connolly, Unwin, *et al.* 2000). In this cohort, it is possible that subtle differences in socio-economic status may influence the results. However, since the cohort is otherwise matched for other factors which influence insulin resistance, particularly body fat and ethnicity, if an effect is present, it is likely to be small.

In Chapter Three, insulin resistance was assessed using fasting measures of insulin sensitivity (HOMA-IR or QUICKI; Figure 3.2); these indices showed

that obese subjects had greater insulin resistance throughout pregnancy and did not demonstrate the same increase in insulin resistance as derived from clamp studies in this chapter. While fasting indices are useful in larger population studies compared to more intensive clamp studies and provide information about fasting beta-cell function, clamp studies have the advantage of providing information about the stimulated state ie responsiveness to insulin. This explains why clamp studies appear to demonstrate a different pattern of insulin sensitivity compared with HOMA-IR values.

In addition to information regarding glucose disposal as an index of insulin sensitivity, when combined with stable isotope tracers, clamp studies provide more detailed information regarding glucose utilisation, endogenous glucose production and rates of lipolysis. These values can be expressed per kg weight but provide different information depending on whether this is expressed per kg total body weight or per kg fat-free mass (FFM), which represents the compartment where most glucose disposal occurs. When Rd glucose is considered by kg total body weight, there is greater glucose disposal under fasting conditions in lean women at all time points. This is likely to be due to lean women having proportionately greater lean mass compared to obese women; since the majority of glucose disposal is in lean, ie fat-free, body mass, lean women will therefore appear to dispose of more glucose per kg total body weight. Although lean women gain proportionately more fat mass in pregnancy compared with obese women, lean women still have a significantly greater proportion of total body weight as lean mass compared to obese women, even in the third trimester. Therefore lean women will still appear to have greater glucose disposal per total body weight throughout pregnancy.

When Rd glucose expressed per kg FFM is considered however, there is increased fasting disposal of glucose in lean and obese women by 36 weeks gestation compared to non-pregnant women and pregnant women at 19 weeks gestation (Figure 4.7). The main contributor to an increase in FFM between 19 and 36 weeks of pregnancy is likely to be the fetoplacental unit as well as increased fluid mass, therefore the increase in Rd glucose from 19 to 36 weeks is likely to represent glucose uptake by the fetoplacental unit. It is similar in both lean and obese women per kg, indicating similar substrate requirements of the fetoplacental unit under both conditions. With administration of insulin, lean women retain sensitivity to insulin-stimulated glucose uptake at 19 weeks where obese women do not, but by 36 weeks, there is little further stimulation of Rd glucose from low dose to high dose insulin.

EGP under fasting conditions is equivalent to Rd glucose under fasting conditions as the system is in isotopic steady state and no exogenous glucose is being administered. EGP reflects hepatic glucose production and is elevated in the third trimester in both lean and obese women as expected when expressed per kg FFM (Figure 4.7). While EGP does not appear to be increased in the third trimester when expressed per total body weight, overall EGP is greater in obese women at this gestation due to greater total body mass. EGP was suppressed by low dose insulin in all groups, with greatest suppression in the most insulin sensitive groups but appeared to be stimulated by high dose insulin, particularly in the most insulin-sensitive groups (lean non-pregnant and lean early pregnancy). This may reflect an artefact of the tracer conditions due to the high volume of exogenous glucose required by very insulin sensitive subjects.

Ra glycerol is indicative of the rate of lipolysis. When this is expressed per kg fat mass, it reflects the lipolytic activity of each unit of fat mass, as adipose is the only tissue which exports glycerol. Ra glycerol is greater in lean compared with obese non-pregnant women under fasting conditions as lipolysis is activated to supply NEFA and glycerol for beta-oxidation and gluconeogenesis; while obese women appear to have a lower rate of Ra glycerol per kg fat mass, approximately 50% that of lean women, they have a much greater overall fat mass, approximately 50% greater than lean women, therefore whole body rate of lipolysis are similar. In pregnancy lean women appear to have much lower fasting Ra glycerol per kg fat mass compared with non-pregnant women, with a further small reduction with advancing gestation whereas obese women do not show such a response to pregnancy. Again, if total body fat mass is taken into consideration, this leads to overall greater total rates of lipolysis in obese compared to lean women throughout pregnancy.

Since Ra glycerol is equal to Rd glycerol under isotopic steady state conditions, this data can also be used to indicate substrate utilisation. Glycerol is primarily taken up by liver and skeletal muscle, the major components of fat-free mass. Therefore where Rd glycerol is expressed per kg FFM, this is indicative of the rate at which glycerol is utilised by these tissues. In Figure 4.8, fasting glycerol utilisation is no different in lean and obese non-pregnant women per kg FFM; and is increased in both lean and obese women by the third trimester. However, while in lean women, there is no increase in glycerol utilisation until 36 weeks gestation, in obese women, the rate of glycerol utilisation begins to increase by 19 weeks gestation, although this is not statistically significant in this cohort.

Accurate measurement of glycerol by GC-MS is notoriously difficult and is reflected by moderately high variances in glycerol measurement. While R_a glycerol is a more accurate measure of lipolysis as this pathway is the only source of glycerol and is not affected by reuptake by adipocse tissue, technical difficulties with the assay can limit the accuracy with which rates of lipolysis can be assessed. An alternative means of assessing lipolysis would be by measurement of the rate of appearance of fatty acids; three moles of fatty acid are released with one mole of glycerol by complete lipolysis of one mole of triglyceride. However, it is not possible to assess fatty acid release using stable isotope tracers in pregnant women as the fatty acid tracers come complexed with albumin which is not approved for use in pregnancy in this context. A further issue is that incomplete lipolysis of triglyceride will also release fatty acids and there is also reuptake of fatty acids by adipocytes following lipolysis. Therefore, while on one hand, R_a glycerol is the better measure of the true rate of lipolysis, it is limited by technical aspects of the assay. On the other hand, measurement of fatty acids is a more reliable assay but is limited by the physiology of fatty acid release. Use of both indices may therefore help to provide more reliable information.

As discussed, in non-pregnant women, total rates of lipolysis appear to be similar when body fat content is accounted for. This is supported by similar levels of fasting NEFA in lean and obese non-pregnant women (Figure 4.9). In pregnancy, it would appear that there is overall total greater lipolysis in obese compared with lean women due to a much greater fat mass, given similar rates of lipolysis per kg fat. This is also supported by an early rise in fasting NEFA and triglyceride in obese pregnant women at 19 weeks gestation, compared with lean women of the same gestation. By 36 weeks, fasting R_a glycerol is similar in obese and lean women, which again would

suggest that total lipolysis is greater in obese women. However, by 36 weeks, fasting NEFA are similar in obese and lean women. This may reflect concomitant greater reuptake of NEFA in obese women, or deposition of NEFA elsewhere as ectopic lipid, resulting in a similar plasma pool of NEFA or it may reflect lack of reliability of Ra glycerol as an accurate assessment of lipolysis in the third trimester.

The lipolytic pathway retained sensitivity to insulin in all subjects. In all groups almost maximal suppression of Ra glycerol was achieved by low dose insulin; lipolysis was not completely suppressed in any group however, this may reflect basal, non-insulin regulated lipolysis. This is consistent with previous studies in pregnant women where maximal suppression was approximately 50% of basal (Sivan, Homko, *et al.* 1999).

In lean women, suppression of lipolysis by low dose insulin was greatest in non-pregnant women, with least suppression in the third trimester of pregnancy. In contrast, in obese women, there were no such changes in response to pregnancy. Utilisation of glycerol was consistently suppressed to a greater extent in lean compared with obese women in pregnancy which may reflect greater basal use of glycerol as a gluconeogenic substrate.

Elevated rates of lipolysis are thought to be important in the third trimester in normal pregnancy to provide maternal sources for gluconeogenesis and triglyceride synthesis and facilitate normal fetal growth and supply of necessary fatty acids to the fetus; reduced third trimester lipolysis is associated with fetal growth restriction (Diderholm, Stridsberg, *et al.* 2006). Maternal triglyceride is hydrolysed by placental lipases and the released fatty acids as well as any circulating maternal NEFA are transported across the placenta by diffusion, and also by selective transport using proteins such as placental Fatty-acyl Binding Protein (pFABP), Fatty Acid Transport

protein (FATP) and Fatty Acid Translocase (FAT) (Campbell, Taffesse, *et al.* 1995; Campbell, Bush, *et al.* 1998; Herrera, Amusquivar, *et al.* 2006; Haggerty, 2010). In obese women, higher rates of maternal lipolysis from earlier on in pregnancy lead to prolonged placental exposure to elevated NEFA as well as triglyceride and may drive elevated supply of NEFA to the fetus. Recent studies have reported altered expression of placental fatty acid transport proteins and lipoprotein lipase activity in obese compared with lean women although this did not correlate with elevated fetal fatty acid concentrations at the time of delivery (Dubé, Gravel, *et al.* 2012). It is possible that prolonged exposure of the fetus to excess NEFA leads to more subtle or long-term changes in fetal metabolism than is reflected by a snapshot of cord blood or birthweight.

From a maternal perspective, prolonged exposure to NEFA may contribute to the pathogenesis of other conditions associated with obese pregnancies, particularly hypertensive disorders. In non-pregnant obese individuals, abnormal NEFA turnover has been suggested to have an independent link with hypertension in addition to effects of insulin resistance and abnormal glucose metabolism (Egan, Hennes, *et al.* 1996; Sarafidis and Bakris, 2010). Similarly, elevated NEFA have been implicated in the pathogenesis of pre-eclampsia, (Hubel, McLaughlin, *et al.* 1996; Vigne, Murai, *et al.* 1997). While there were no significant differences between lean and obese subjects in blood pressure in this cohort, there were significant elevations in both systolic and diastolic blood pressure observed in a larger cohort, (Chapter 3, Table 3.1). Although this was still within normal limits of blood pressure, it may represent a subtle right-shift in the distribution of blood pressure in the obese pregnant population, which would contribute to the higher proportion of obese women diagnosed with hypertensive disorders of pregnancy.

Both inflammatory cytokines and altered adipokine expression have been implicated as drivers of altered maternal metabolism. Consistent with this, we have shown elevated circulating IL-6 in obese compared with lean women, greatest in the third trimester, as well as elevated leptin throughout pregnancy. In adipose tissue, in lean women, there was elevated expression of pro-inflammatory cytokine transcripts in pregnant compared with non-pregnant women; in contrast, pro-inflammatory cytokine transcripts were broadly elevated in both pregnant and non-pregnant obese women. TNF α in particular has been implicated as having a role in the insulin resistance of pregnancy (Kirwan, Haugel-De Mouzon, *et al.* 2002), this is supported by our observation of increased TNF α transcript in adipose tissue of lean pregnant women compared with non-pregnant women. However, it may be that in the context of obesity, TNF α expression is already maximal, given that there is no further increase in response to pregnancy in obese pregnant women.

Other increases in the expression of pro-inflammatory cytokines observed in lean pregnant women may reflect a response to expansion of adipose tissue mass as lean women lay down fat stores in pregnancy. MCP-1 mRNA in particular is elevated in lean pregnant compared with non-pregnant women, with a similar though smaller magnitude increase in IL-6 mRNA levels. However, there was a similar though not statistically significant increase in IL-6 mRNA levels in obese pregnant compared with non-pregnant women, suggesting that there may be an additional effect of pregnancy rather than simply fat mass expansion, given that obese women tend to put on less fat mass compared with lean women.

With respect to adipokines, circulating levels of leptin are increased in obese compared with lean women, as well as increasing in lean pregnant compared

with lean non-pregnant women. Transcript levels of leptin are also increased in adipose tissue of lean women in pregnancy to a similar level as that observed in obese pregnant women. This would be consistent with expected changes in adipose tissue mass. In particular, at 36 weeks there is no difference in transcript levels of leptin between lean and obese pregnant women, which is in keeping with our unexpected findings in Chapter 3 (Figure 3.3). However in this study, we also found that lean women appeared to increase circulating adiponectin in response to pregnancy; this is in contrast to data from our larger cohort in Chapter 3 (Figure 3.4) and to previous published studies, which showed maintenance of plasma adiponectin from pre-pregnancy to early pregnancy, but a significant reduction in the third trimester, associated with reduced white adipose tissue adiponectin mRNA levels (Catalano, Hoegh, *et al.* 2006). Additionally, there did not appear to be any difference in total adiponectin levels between lean and obese non-pregnant women, where a reduction in obese women would be expected. However, the pattern of adiponectin transcript levels in adipose tissue is more consistent, with reduced mRNA levels in the tissues of obese compared to lean women. Broadly, obese women appear to have lower plasma adiponectin than lean women, which is consistent with the literature, despite an apparent lack of difference in the non-pregnant group, which may be an effect of the small numbers and therefore caution is needed in interpretation. Data from the larger cohort in Chapter three is more consistent with previously reported adiponectin levels in pregnancy.

Within adipose tissue, there were some differences between obese and lean pregnant women in transcript levels of genes regulating lipid synthesis and storage. Due to the small numbers in this cohort, few of these differences reached statistical significance, although there were significant differences

between lean and obese women in some key genes controlling lipid synthesis and insulin regulation, particularly decreased stearoyl desaturase-1 (SCD1), fatty acid synthase (FASN) and insulin receptor substrate 1 (IRS1) (Figure 4.16), possibly reflecting an earlier suppression of lipid storage in obese women compared with lean. For transcripts where statistical significance was not reached, there was a similar pattern of reduction in transcript level at an earlier gestation in obese compared to lean women, eg. malic enzyme 1 (ME1), which is important for generation of NADPH for lipid synthesis. It is tempting to speculate that these expression patterns are consistent with an earlier shift in the adipose tissue of obese subjects away from lipid storage in early pregnancy, while this shift occurs later in lean subjects. However, a larger cohort of samples would be required to verify this, potentially also supported by assessment of enzyme quantity and function at protein level as protein function does not always correlate well with transcript levels.

We have shown that lean women have a relatively late decrement in insulin sensitivity and other metabolic adaptations to pregnancy such as increase in triglyceride and NEFA, whereas obese women appear to be in this state pre-pregnancy and do not mount much further response to pregnancy. This is important in two respects: firstly, it implies that the metabolic adaptations of pregnancy may be overridden by the pre-existing effects of obesity or secondly, that there may be a limit beyond which further development of insulin resistance is not possible or desirable.

With respect to pre-gravid obesity over-riding the metabolic demands of pregnancy, this may affect some but not all aspects of maternal metabolism. There are still some differences in the metabolic profile of obese pregnant women compared with non-pregnant obese women, particularly with respect to early rises in triglyceride and NEFA. However, when compared

with lean women, these changes occur at an earlier gestation. This may represent utilisation of what little reserve such patients have to respond to the various hormonal stimuli of pregnancy. The consequence of this is prolonged exposure of both the maternal and fetal systems to an environment which normally only exists in the third trimester.

Following on from this, why does the maternal environment not simply continue to deteriorate, and become more insulin-resistant, with further hypertriglyceridemia? To avoid this further deterioration, there may be activation of counter-acting pathways. Evidence in support of this hypothesis includes increased expression of circulating anti-inflammatory cytokines in the plasma of obese pregnant women (Figure 4.12). Elevated IL-1Ra has been reported in both murine and human obesity, both circulating cytokine and white adipose tissue expression (Meier, Bobbioni, *et al.* 2002; Juge-Aubry, Somm, *et al.* 2003). In pregnant rats, IL1-Ra levels were no different to that of non-pregnant animals although their responses to injected lipopolysaccharide differed (Fofie, Fewell, *et al.* 2004; Ashdown, Poole, *et al.* 2006); in human pregnancy however, an increase in IL-1Ra has been reported (Østensen, Förger, *et al.* 2004) including a potential link between fasting glucose, BMI and birthweight (Friis, Frøslie, *et al.* 2010). A further rise in IL-1Ra in obese pregnant women may represent a potential mechanism to attenuate the adverse effects of excess pro-inflammatory cytokines.

The concept of allostatic regulation of metabolism and allostatic load has been applied to obesity to account for the pathological processes observed in an obese non-pregnant population. Allostasis is the maintenance of normality through change and involves activation of compensatory mechanisms during periods of internal or external stress to promote adaptability (McEwen, 1998). Where these mechanisms are chronically

activated or inactivated, this leads to allostatic load, which contributes to pathological changes and disease progression (McEwen, 1998). Normal pregnancy could be viewed as a temporary state of increased demand and change to maternal physiology, where certain pathways are activated to provide the required protection of maternal physiology during pregnancy, but which are then returned to normal after pregnancy is complete. In obesity, if some of these pathways are already maximally or constitutively activated, then this may result in an inability to mount the normal response to pregnancy (Power & Schulkin, 2012). Therefore some of the features we observe in obese pregnant women may be secondary to activation of adaptive responses and ultimately a limitation of plasticity within their physiology, where a normal allostatic response becomes pathological due to a pre-existing load.

4.5. Conclusions

In this chapter it has been shown that obese pregnant women have profound insulin resistance, elevated triglyceride, NEFA, pro-inflammatory cytokines and dysregulated adipokine profile compared with lean pregnant women. We expected to find exaggeration of such metabolic features in these women as a result of the presence of the dual stressors of pre-gravid obesity and pregnancy; however, we instead found that by the third trimester, there were fewer differences between lean and obese women, particularly with respect to adipose tissue. Therefore, it may be that the adverse outcomes associated with obese pregnancies are due to duration or timing of exposure to an adverse metabolic profile rather than severity of exposure per se, and may reflect a greater pre-existing allostatic load in obese pregnant women compared with lean women. Therapeutic interventions may therefore be of most benefit if targeted to early pregnancy, though ideally pre-pregnancy, with the aim of improving maternal metabolic status at earlier gestations and reducing fetal exposure. However, the difficulty with such interventions would be in identification of an optimal target which would permit improvement in outcome without restricting the degree of adaptation which is required for a healthy pregnancy.

The consequences of prolonged duration of exposure to a period of insulin resistance in obese women might be reflected in part in altered adipose tissue distribution with excess ectopic lipid in liver and skeletal muscle as well as in a differing pattern of adipose tissue accumulation in pregnancy between lean and obese women; in the next chapter, magnetic resonance imaging techniques are employed to examine both of these features in the third trimester.

Chapter 5

AMPOP study: Adipose distribution in the third trimester assessment using Magnetic Resonance Imaging

5.1. Background

Adipose tissue distribution patterns have been associated with variations in risk of cardiovascular disease, notably an increased risk of insulin resistance, metabolic syndrome and type II diabetes mellitus in subjects with predominantly centrally distributed obesity (Lapidus, Bengtsson *et al.* 1984; Kissebah and Krakower. 1994; Canoy, Boekholdt *et al.* 2007; Fox, Massaro, *et al.*, 2007; Demerath, Reed *et al.*, 2008). This has led to the ‘portal’ theory of central obesity, where increased exposure of the liver to elevated levels of inflammatory cytokines, NEFA and other metabolites from omental adipose is thought to lead to hepatic insulin resistance (Kabir, Catalano, *et al.* 2008; Catalano, Stefanovski *et al.* 2010; Rytka, Wueest *et al.* 2011). However, the evidence for this being the dominant mechanism to account for the observed associations between adipose tissue distribution and metabolic disease is not strong.

Obesity is also associated with accumulation of ectopic lipid outwith specific adipose tissue depots typically in liver (Bozzetto, Prinster *et al.* 2010) and skeletal muscle (Goodpaster, Theriault, *et al.*, 2000), and particularly in association with abdominal obesity (Jakobsen, Berentzen *et al.*, 2007) and is thought to reflect saturation of the storage capacity of the adipose depot. However, in patients with lipodystrophy, there is liver lipid accumulation and development of severe insulin resistance despite lack of subcutaneous

adipose tissue (Reitman, Arioglu *et al.*, 2000). In animal models of this condition, transplantation of subcutaneous adipose reverses the insulin resistance (Gavrilova, Marcus-Samuels *et al.*, 2000) as does treatment with leptin in humans (Petersen, Oral *et al.*, 2002). Therefore it is not simply the presence of excess adipose, or more specifically excess subcutaneous adipose, which drives ectopic lipid accumulation.

With respect to obesity, at any given level of obesity, there is a wide variance in insulin sensitivity (Ferranini, Natali *et al.*, 1997) and the severity of observed hepatic steatosis is thought to reflect the degree of underlying insulin resistance rather than body fat content alone (Seppälä-Lindroos, Vehkavaara *et al.*, 2002). The relationship between insulin resistance and ectopic lipid accumulation is less clear, ie. whether lipid accumulation is a cause or a consequence of insulin resistance. Hepatic lipid accumulation is associated with elevated inflammation and oxidative stress (Day and James, 1998). Thus, liver lipid accumulation initiates a cycle of worsening IR and enhanced lipid deposition, culminating in hepatic inflammation, fibrosis and hepatocellular damage. Clinically this manifests as non-alcoholic steatohepatitis, with deranged liver enzymes and hepatic dysfunction. Similarly, Intramyocellular lipid accumulation is associated with impaired IRS1 and PI3K action in human males (Virkamäki, Korshennikova *et al.*, 2001).

The relative contributions to the metabolic requirements of pregnancy of the pattern of fat accumulation or of the different adipose depots are not clear. We have already shown that obese women tend to gain less weight in pregnancy than lean women (Chapter 3 & 4), which is consistent with previous studies showing that lean women tend to accumulate adipose

peripherally in contrast to obese women as assessed by skinfold thickness (Ehrenberg, Huston-Presley *et al.* 2003). However, there have not been any studies of intra-abdominal adipose tissue distribution to date.

Similarly, hepatic and skeletal muscle lipid content have not previously been quantified in healthy pregnant women, although the rare condition of acute fatty liver of pregnancy is recognised (Moore, 1956; Ch'ng, Morgan, *et al.* 2002; Knight, Nelson-Piercy, *et al.* 2008). The aetiology of this condition is not known but it is associated with rapid fat accumulation in the liver, in conjunction with elevated transaminases and hepatocyte damage. We have already shown in Chapter 4 that in the third trimester, lean women are significantly more insulin resistant than in the first half of pregnancy. Whether this manifests in hepatic lipid accumulation is not known. We have also shown that obese pregnant women have prolonged exposure to insulin resistance compared with lean women; this might be expected to be reflected by excess ectopic accumulation of lipid.

Assessment of body fat distribution in pregnancy is technically challenging, particularly where the visceral component is concerned, and requires the use of imaging techniques. To date, no other studies have made use of magnetic resonance imaging to assess body fat distribution in pregnancy, although this is a recognised approach in the non-pregnant population. It has the obvious advantage of avoiding the use of ionising radiation necessary with CT imaging and provides a much clearer definition of tissue margins than ultrasound, a widely used imaging modality in obstetrics. Similarly, the use of MRI also provides an opportunity to use non-invasive methods to

quantify ectopic lipid accumulation, in a population in whom the gold standard liver biopsy is clearly neither safe, practical or acceptable.

We hypothesised that:

1. MR imaging is practical to conduct in pregnancy in the third trimester at 3T.
2. MR imaging can be used to quantify intra-abdominal fat distribution.
3. Hepatic and skeletal muscle lipid content is elevated in obese compared with lean pregnant women in the third trimester.
4. An increase in intra-abdominal adipose tissue accounts for the majority of the difference in adipose tissue mass between lean and obese women.

In this study we aimed to:

1. Optimise an imaging protocol for measurement of (a) intra-abdominal adipose distribution and (b) hepatic and skeletal muscle lipid content in pregnant women in the third trimester.
2. Quantify and compare adipose tissue distribution in lean and obese pregnancy women at 36 weeks gestation.
3. Quantify and compare hepatic and skeletal muscle lipid in lean and obese pregnant women at 36 weeks gestation.

5.2. Methods

5.2.1. Patient recruitment, exclusion and inclusion criteria

Pregnant women were recruited from the Metabolic Antenatal Clinic and control cohort as described previously and were imaged at approximately 36 weeks gestation. Lean women had a booking BMI between 20.0 and 25.0 kg/m²; obese women had a booking BMI > 40.0 kg/m².

All women had healthy singleton pregnancies, a normal OGTT at 28 weeks and no contra-indications to MR scanning. Demographic characteristics are shown in Table 5.1. A fasting blood sample was obtained at the study visit for quantification of glucose, and anthropometric measurements made, including estimation of fat mass using bioelectrical impedance.

5.2.2. Scanning protocol

Patients were imaged at the Clinical Research Imaging Centre at the Royal Infirmary of Edinburgh on a Siemens Magnetom Verio 3T Whole Body MRI scanner (Siemens Medical, Erlangen, Germany). Patients were positioned in the scanner in a left lateral position to prevent aorto-caval compression. MRI data was obtained using a combination of body matrix and spine matrix coil elements. The body matrix coil was positioned over the maternal liver and thigh. Aural protection was provided by use of ear plugs and headphones. Contact was maintained with the scanning staff at all times through the use of an intercom. Heart rate and SpO₂ were continuously monitored during the

scan process; blood pressure was recorded prior to commencing scanning and every 10 minutes thereafter.

Scanning was carried out by Annette Cooper (radiologist) and Scott Semple (MR physicist). Image analysis was carried out by the investigator (SB), Carolyn Chiswick (clinical research fellow) and Calum Gray (physicist). Landmarking of images was carried out by SB, CG and Jane Walker (consultant radiologist).

5.2.3. Scanning sequences

MRI and MRS were acquired with a combination of body matrix and spine matrix elements. Two dimension (2D) multi-slice FLASH images were acquired axially central to the right lobe of the maternal liver with water and lipid signals in and out of phase, using echo times of 2.46, 4.92, and 8.61ms. MRI and MRS were also acquired in maternal right quadriceps.

5.2.4. Quantification of intra-hepatic and skeletal muscle lipid concentration

5.2.4.1. ^1H -MR Spectroscopy

^1H MR spectroscopy (^1H -MRS) is a technique which can be used to detect and quantify specific metabolites in a region of interest based on differing resonant frequencies of protons depending on the structure of the molecule they are present in. Thus different metabolites, in this case triglyceride, are detectable in tissue by their unique position in the generated frequency

spectrum. ^1H -MRS can be used to quantify intracellular lipid as well as the total lipid content in a region of interest (Szczepaniak, Nurenberg *et al.* 2004; Springer, Machann *et al.*, 2010)

^1H proton MRS PRESS single voxel spectra were obtained in the right lobe of the liver remote from any large vessels or subcutaneous adipose using an echo time of 30 ms and a voxel size of 3cm^3 . Spectra were also obtained in the right quadriceps muscle using the same echo time and a voxel size of 2cm^3 . Exemplar hepatic spectra are shown in Figure 5.1 below.

Spectra were obtained with and without water suppression, and lipid concentration was calculated from the water-suppressed acquisition using the spectroscopy analysis tool JMRUI. This step is necessary in tissues with low lipid content, as in such tissues the water peak of the spectrum is much larger and wider than the lipid peak, which is thus more difficult to resolve.

Figure 5.1 Representative hepatic spectra from lean and obese subjects

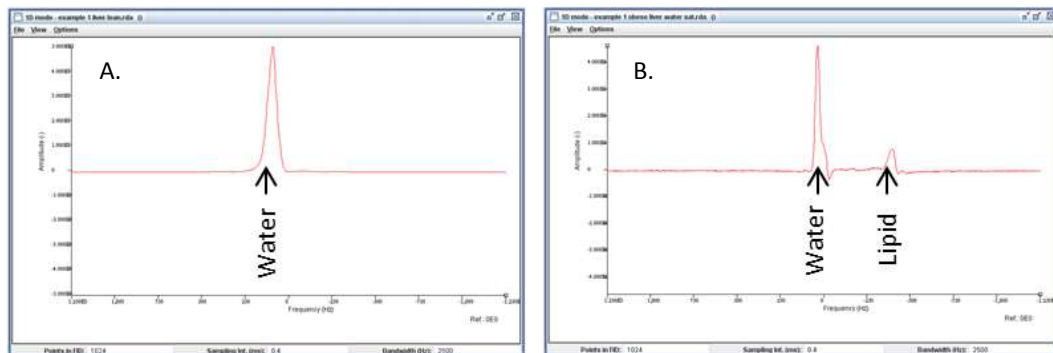


Figure 5.1 Representative hepatic spectra from a lean subject (A) (BMI at 36 weeks 26.3kg/m^2) and obese (B) (BMI at 36 weeks gestation, 43.7 kg/m^2).

5.2.4.2. In- and out-of-phase imaging

In some subjects, it was not possible to obtain satisfactory hepatic ^1H -MRS data. This occurred most commonly in subjects with the highest BMI, in whom there were issues with loading of the subject within the bore of the scanner, limiting spectroscopy data acquisition. This occurred for two reasons: firstly, significant distortion of the magnetic field led to difficulty with a process required to compensate for such lack of homogeneity (shimming); secondly, the region of interest (eg. liver) was physically further away from the coil therefore less signal was detected.

Intrahepatic lipid signal contribution was therefore also calculated by the Dixon method through subtraction of in and out of phase images (2.46ms and 8.61ms), and T_2^* decay during this time corrected using the two in-phase images (2.46ms and 4.92ms) according to a well-established protocol (Hussain, Chenevert *et al.* 2005; Irwan, Edens *et al.* 2007; Kim, Taksali, *et al.* 2010).

5.2.5. Assessment of Abdominal Adipose

5.2.5.1. Data acquisition

To define subcutaneous and intra-abdominal fat, a 3D T1-weighted VIBE sequence was acquired axially through the liver with in and out phase images, and lipid signals defined using a combination of the in and out of phase images with a semi-automated thresholding technique employed using the commercial software SliceOmatic™ (TomoVision, Quebec, Canada).

Adipose tissue appears bright on T1-weighted images. Regions of interest with attenuation above an investigator-defined threshold were coloured to delineate intra-abdominal adipose (red) and subcutaneous adipose (green). The area of the coloured regions was extracted, converted into a unit of volume (mm^3) and expressed as a percentage of the total abdominal volume being examined. Intra-abdominal adipose was defined as all adipose tissue below the abdominal wall musculature; this includes omental, mesenteric and paranephric deposits but does not distinguish between intra- and retro-peritoneal deposits. Subcutaneous adipose was defined as all adipose inferior to skin but outwith the abdominal cavity. Adipose tissue depots which were not definably subcutaneous but were obviously outwith the abdominal cavity were coloured blue and defined as paraspinal adipose. Where breast tissue was in the field of view, this was excluded from analysis. Example images of lean and obese subjects are shown in Figure 5.2.

Adipose tissue volumes were calculated using the extracted areas of the regions of interest (mm^2) multiplied by the width of each slice (2mm); this was expressed as a volume in litres by dividing the volume in mm^3 by 1×10^6 . Adipose tissue mass was then be calculated using an estimate of adipose tissue density of 0.9kg/L.

Figure 5.2 Representative abdominal slices from lean and obese subjects

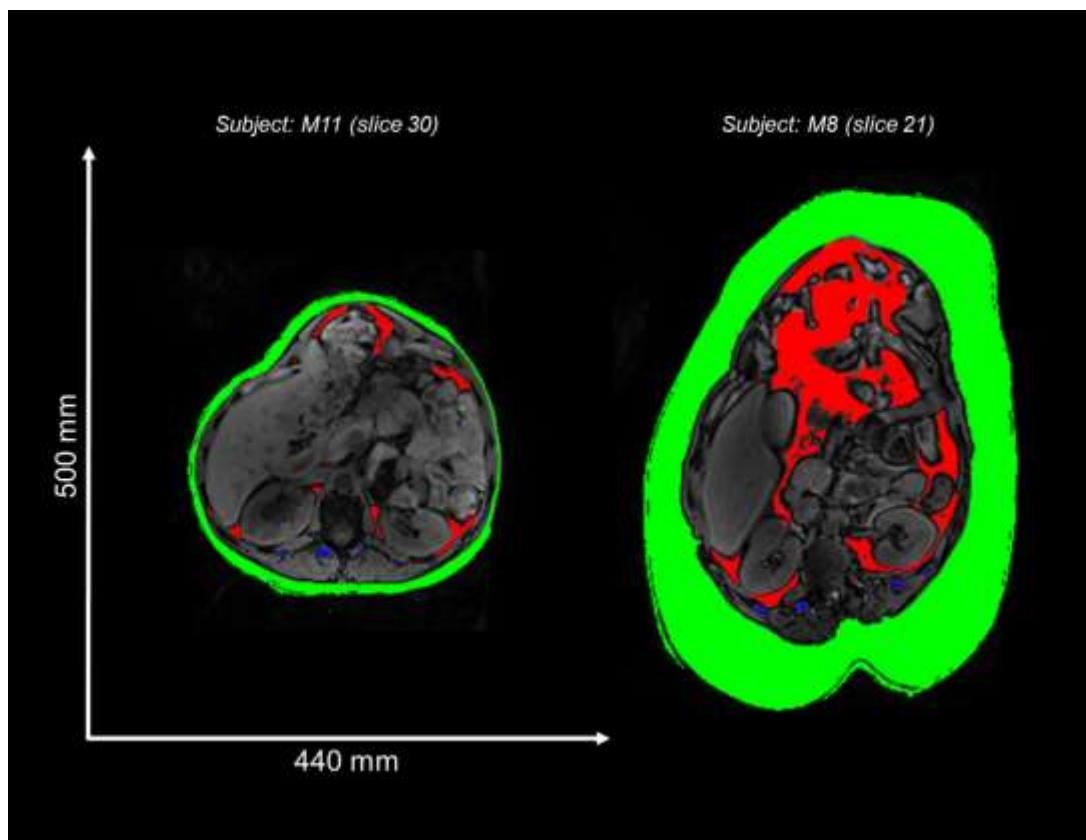


Figure 5.2 Representative abdominal slices from lean (A) (BMI at 36 weeks, 23.4 kg/m^2) and obese (B) (BMI at 36 weeks 43.7 kg/m^2).

Green, subcutaneous adipose; Red, intra-abdominal adipose; Blue, paraspinal adipose.

5.2.5.2. Region selection

A multi-slice approach was used as described in Chapter Two (2.7.3). Briefly, MR imaging facilitates acquisition of 2D image data as well as single or multiple slice 3D image data. Single slice data has the advantage of being rapid to acquire and analyse, but is limited in how representative it is of the region of interest. Multi-slice data takes longer to acquire and analyse but is thought to be more representative. Images were landmarked by the investigator (SB) and a consultant radiologist (Dr Jane Walker). The left renal pelvis was identified in all patients. Regions of adipose tissue were marked as described above in 20 x 2mm slices cranial to this level.

5.2.5.3. Reproducibility

Intra-observer variability was assessed by the same observer (SB) defining all three regions on 5 slices from 9 subjects ($n = 45$ comparisons per depot) on two separate occasions. Inter-observer variability was assessed by comparison of data from 21 slices from 10 patients ($n = 210$ comparisons for 3 depots) by two independent observers (SB and CC).

For each set of paired data, two approaches were used to assess agreement. Firstly, readings were plotted pairwise, M1 vs M2, where M1 was the initial measurement and M2 was the repeated measure (Figure 5.4 and 5.6). The line of agreement was also plotted, representing expected values for M2 if there were 100% agreement between M1 and M2. Linear regression was performed for the actual data; the closer the slope of the actual fit to 1, the

better the agreement between the 2 measures. M1 and M2 were correlated using Pearson correlation.

Secondly, Bland Altman plots were constructed for each set of data (Bland and Altman, Lancet, 1986); this is a plot of the mean of M1 and M2 against the difference between them (M1-M2) (Figure 5.5 and 5.7). Ninety-five per cent limits of agreement were calculated as follows:

$$\text{Upper 95\% limit of agreement} = \text{mean difference} + 2SD$$

$$\text{Lower 95\% limit of agreement} = \text{mean difference} - 2SD$$

5.3. Results

5.3.1. Patient Demographic Characteristics

Demographic characteristics of participants are shown in Table 5.1. Details are given for subjects whose data was used for the main analysis, excluding subjects scanned as part of protocol development.

The mean age of women in the obese cohort was slightly lower than the lean cohort (28.4 ± 1.1 vs 32.5 ± 1.4 years, $p = 0.04$). While this is statistically significant, this four year difference does not represent a clinically significant difference in age. This perhaps represents the socioeconomic circumstances of the population of women from whom recruitment was drawn. There were no differences between the groups in gestation at study visit or parity.

Table 5.1 Demographic characteristics of study cohort

	Lean (n = 10)	Obese (n = 10)	p
Age (years)	32.5 ± 1.4	28.4 ± 1.1	0.04
Height (m)	1.65 ± 0.02	1.63 ± 0.01	0.43
Weight (kg)			
Baseline	61.95 ± 1.6	117.9 ± 4.7	< 0.0001
Study visit	73.3 ± 2.2	123.3 ± 4.9	< 0.0001
p, Visit 1 vs Visit 2	< 0.0001 ^a	0.002 ^a	
BMI (kg/m ²)			
Baseline	22.8 ± 0.4	44.2 ± 1.3	< 0.0001
Study visit	27.0 ± 0.8	46.2 ± 1.3	< 0.0001
p, Visit 1 vs Visit 2	< 0.0001	0.002	
Median gestation (weeks+days)			
Baseline	15+1	20+3	0.07
Study visit	36+6	36+3	0.07
Parity (Nulliparous/Total n)	6/10	3/10	0.37
SBP at 36 weeks (mmHg)	108.9 ± 2.4	124.4 ± 3.2	0.001
DBP at 36 weeks (mmHg)	64.0 ± 2.7	73.4 ± 2.1	0.01
Mean Δ weight (kg)	11.4 ± 1.4	5.3 ± 1.2	0.004
Fat mass at 36 weeks (kg)	24.7 ± 1.3	60.8 ± 3.6	< 0.0001
Fasting glucose at 36 weeks (mmol/L)	4.1 ± 0.1	4.5 ± 0.2 (n=6 ^b)	0.03
Mode of delivery			
Vaginal delivery	5	6	
LUSCS	5	4	
Total	10	10	1.0 ^c
Median gestation at delivery (weeks+days)	40+4	40+4	0.60
Birthweight (g)	3665 ± 113	3963 ± 194	0.18

Data are expressed as mean ± SEM unless otherwise specified.

Groups are compared using Students' unpaired t-test unless otherwise specified.

- Data were compared between visit 1 and visit 2 using paired Students' t-test
- Fasting blood glucose values at 36 weeks were only available for 6 of the women in the obese cohort as 4 women did not attend for blood sampling at this gestation.
- Delivery data were compared using Fisher's exact test. Vaginal delivery includes spontaneous vaginal deliveries and instrumental vaginal deliveries.

5.3.2. Total abdominal volumes

Total abdominal volumes within the 20-slice region analysed are shown in Figure 5.3. At 36 weeks gestation, obese women had significantly greater abdominal volumes compared with lean women in the same region. (6.63 ± 0.3 vs 3.78 ± 0.2 litres, $p < 0.0001$).

Figure 5.3 Total abdominal volumes

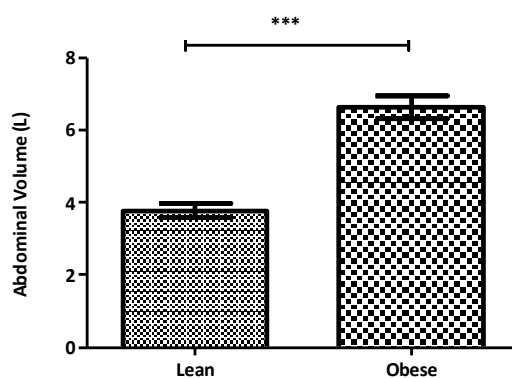


Figure 5.3 Total abdominal volumes within 20 x 2mm slice region of interest. Data are expressed as mean \pm SEM; $n = 10$ per group. *** $p < 0.0001$ Data are compared using Students' unpaired t-test.

5.3.3. Reproducibility of measurements

Following repeated measurements by the same investigator (intra-rater) and of the same images by 2 investigators (inter-rater), correlative analysis was performed (Figure 5.4 and Figure 5.6). Measurements were highly correlated for pairs of measurements for each depot, Pearson r was greater than 0.97 for each depot.

Bland-Altman analysis was carried out to illustrate the agreement between the pairs of measurements (Figure 5.5 and 5.7). Overall, on repeated measurement by both the same investigator and by another investigator there was a tendency to overestimate fat mass by up to approximately 5cm³.

Figure 5.4 Intra rater reproducibility: correlative analysis

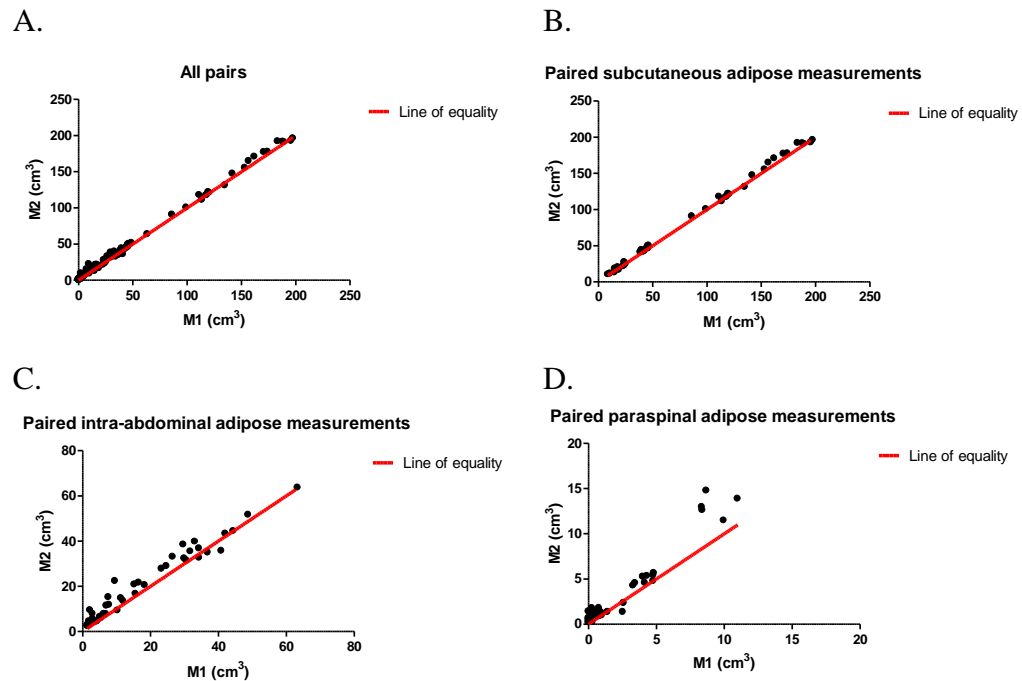


Figure 5.4 Initial (M1) and repeated (M2) measurements of adipose volume per slice were plotted (cm³). The line of equality in each plot has slope = 1, where M1 = M2.

- (A) All pairs of data; n = 135. Slope = 1.00 ± 0.005 . Pearson $r = 0.99$
- (B) Paired SC data; n = 45. Slope = 1.02 ± 0.006 . Pearson $r = 0.99$
- (C) Paired intra-abdominal data; n=45. Slope = 0.97 ± 0.03 . Pearson $r = 0.98$
- (D) Paired paraspinal data; n = 45. Slope = 1.29 ± 0.05 . Pearson $r = 0.97$

Figure 5.5 Intra-rater reproducibility: Bland Altman analysis

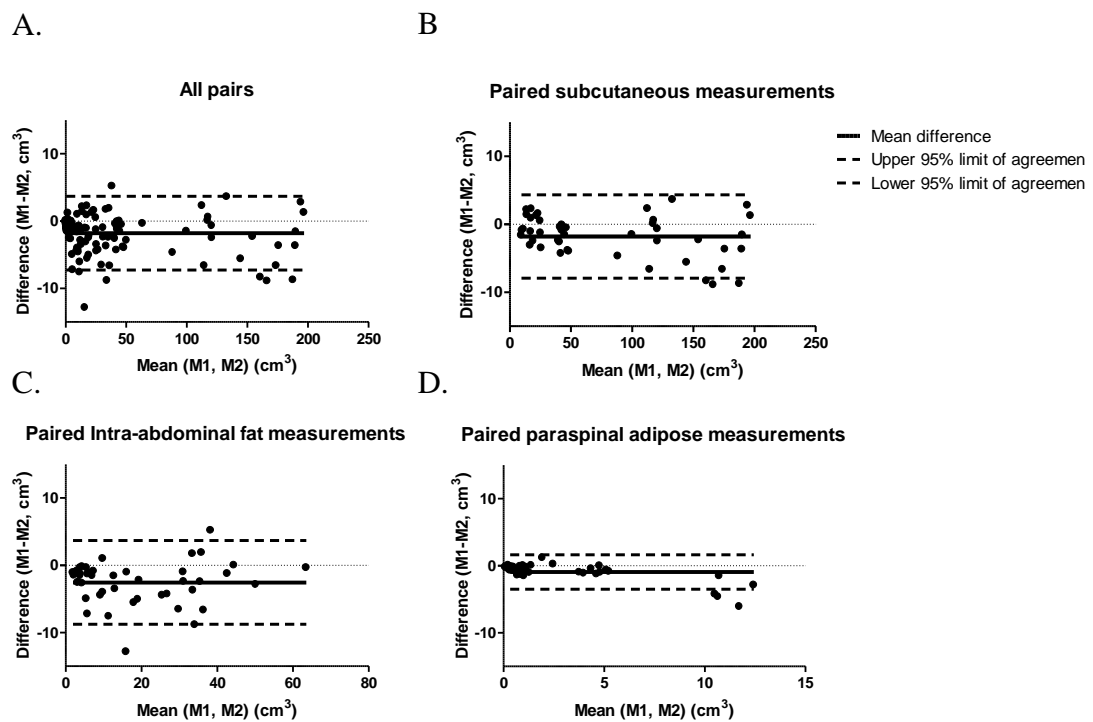


Figure 5.5. Bland Altman plots of paired measurements.

- (A) All pairs of measurements ($n = 130$); Mean difference (Lower – upper 95% limits of agreement) = -1.79 ($-7.26 - 3.68$) cm³.
- (B) Paired subcutaneous measurements ($n = 45$); Mean difference (Lower – upper 95% limits of agreement) = -1.79 ($-7.91 - 4.34$) cm³.
- (C) Paired intra-abdominal measurements ($n = 45$); Mean difference (Lower – upper 95% limits of agreement) = -2.55 ($-8.77 - 3.68$) cm³.
- (D) Paired paraspinal measurements ($n = 45$); Mean difference (Lower – upper 95% limits of agreement) = -0.91 ($-3.48 - 1.65$) cm³.

Figure 5.6 Inter rater reproducibility: correlative analysis

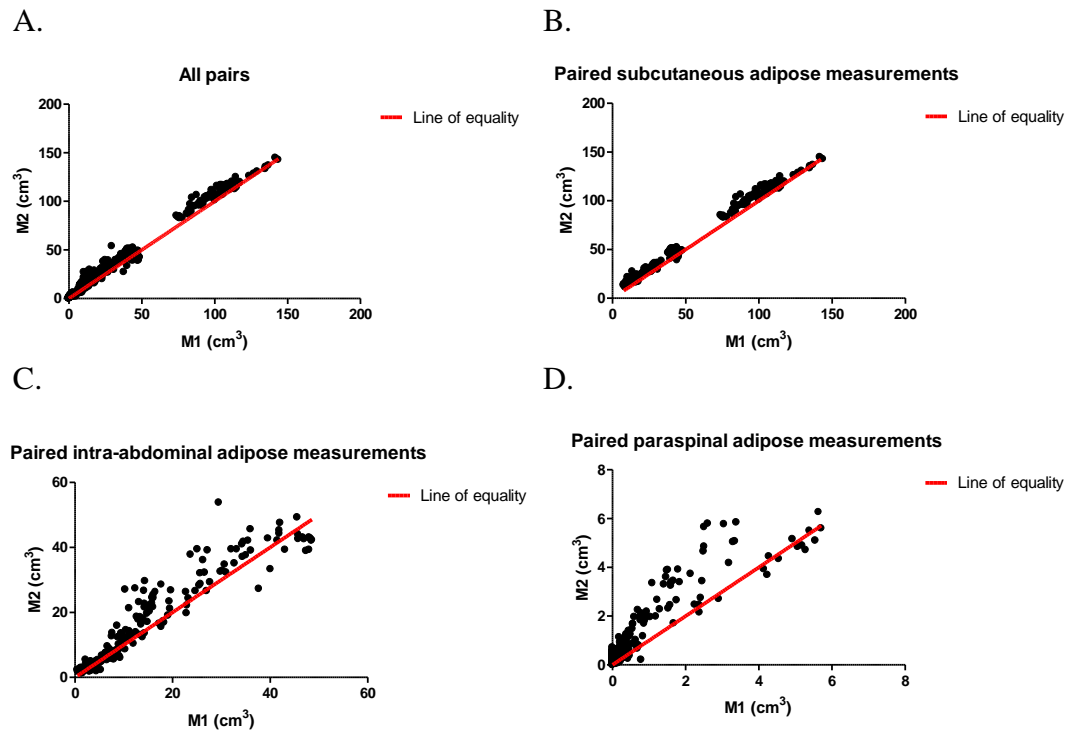


Figure 5.6 Initial (M1) and repeated (M2) measurements of adipose volume per slice were plotted (cm³). The line of equality in each plot has slope = 1, where M1 = M2.

- (A) All pairs of data; n = 672. Slope = 1.04 ± 0.004 . Pearson $r = 0.99$
- (B) Paired SC data; n = 231. Slope = 1.01 ± 0.006 . Pearson $r = 0.99$
- (C) Paired intra-abdominal data; n=231. Slope = 1.02 ± 0.02 . Pearson $r = 0.95$
- (D) Paired paraspinal data; n = 210. Slope = 1.09 ± 0.03 . Pearson $r = 0.91$

Figure 5.7 Inter-rater reproducibility: Bland Altman analysis

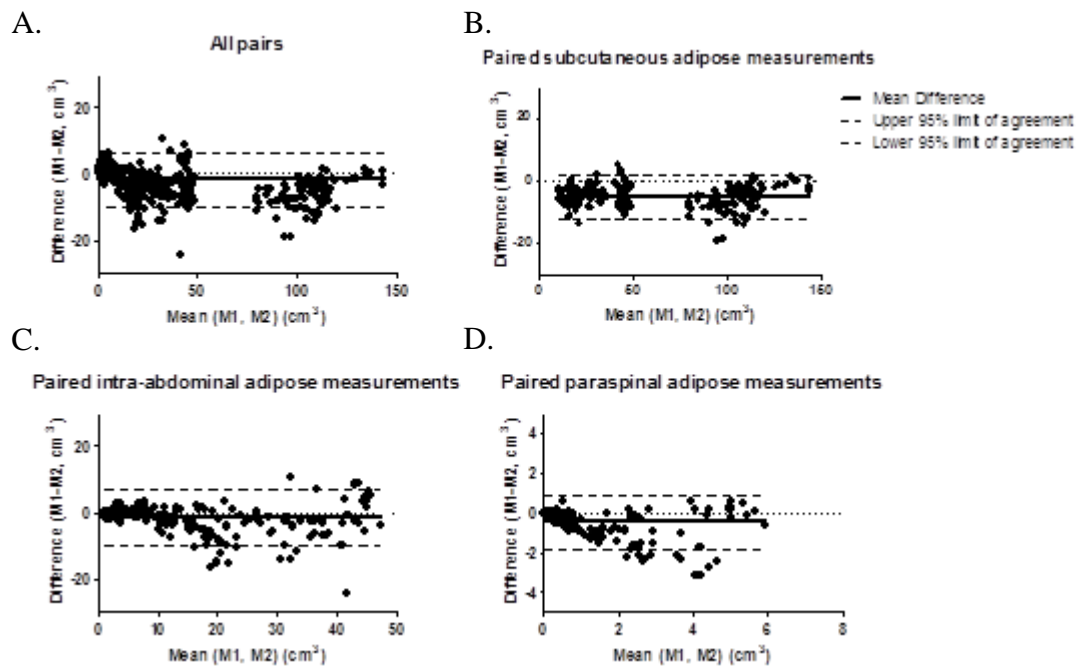


Figure 5.7. Bland Altman plots of paired measurements.

- (A) All pairs of measurements ($n = 672$); Mean difference (Lower – upper 95% limits of agreement) = -1.80 ($-10.16 - 6.56$) cm^3 .
- (B) Paired subcutaneous measurements ($n = 231$); Mean difference (Lower – upper 95% limits of agreement) = -4.9 ($-11.9 - 2.0$) cm^3 .
- (C) Paired intra-abdominal measurements ($n = 231$); Mean difference (Lower – upper 95% limits of agreement) = -1.5 ($-10.0 - 6.9$) cm^3 .
- (D) Paired paraspinal measurements ($n = 210$); Mean difference (Lower – upper 95% limits of agreement) = -0.4 ($-1.8 - 0.9$) cm^3 .

5.3.4. Subcutaneous, intra-abdominal and paraspinal fat masses

Fat masses in the subcutaneous, intra-abdominal and paraspinal adipose depots are shown in Figure 5.8. Obese women had significantly greater fat masses in all 3 depots compared with lean women (subcutaneous, 2308 ± 197 vs 539.9 ± 68.8 g, $p < 0.0001$, Figure 5.8A; intra-abdominal, 534.3 ± 64.8 vs 128.8 ± 29.9 g, $p < 0.0001$, Figure 5.8B; paraspinal, 57.1 ± 15.6 vs 6.7 ± 1.8 g $p = 0.005$, Figure 5.8C).

Figure 5.8 Depot-specific fat mass estimation

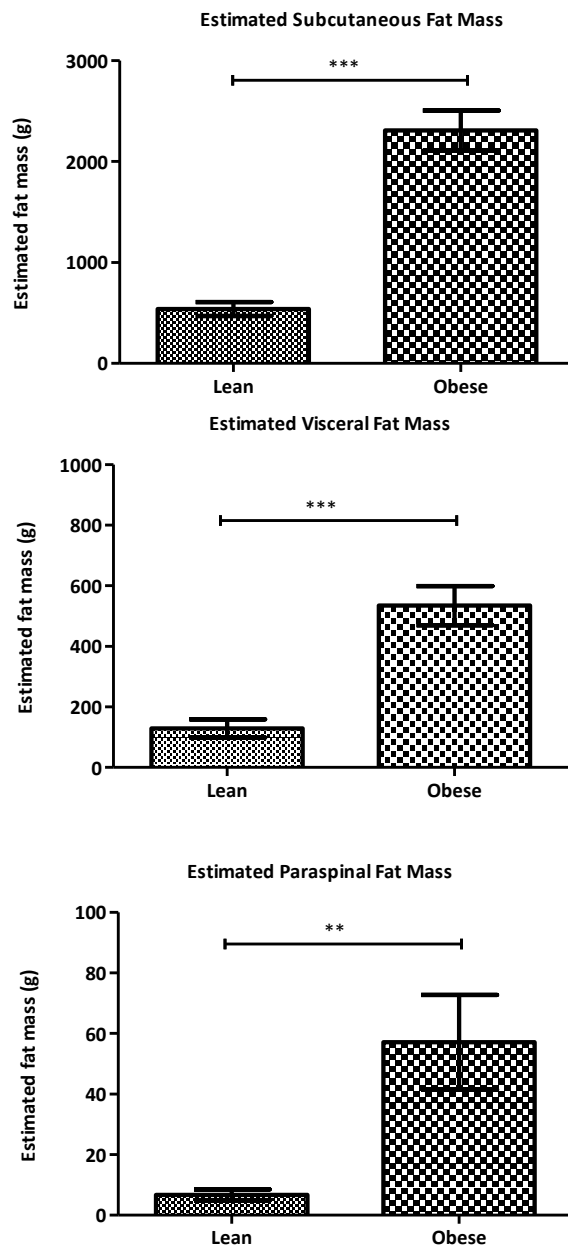


Figure 5.8

Fat mass in subcutaneous, intr-abdominal and paraspinal adipose depots.

Data are expressed as mean \pm SEM; n = 10 per group. ** p < 0.01 *** p < 0.0001

Data are compared using Students' unpaired t-test.

5.3.5. Intra-hepatic and Intra-myocellular fat fraction

Hepatic fat fraction was calculated using the Dixon method as described and expressed as a percentage of total liver volume. There was no significant difference between obese and lean women in hepatic fat fraction (1.2 ± 0.3 vs 1.6 ± 0.4 %, $p = 0.34$, Figure 5.9).

Intramyocellular lipid fraction was calculated using ^1H -MR spectroscopy from a 2cm^3 voxel placed in the right quadriceps muscle as described. Obese women had significantly greater intramyocellular lipid compared with lean women at 36 weeks gestation (10.7 ± 2.1 vs 3.1 ± 0.8 %, $p = 0.003$, Figure 5.10).

Figure 5.9 Hepatic fat fraction in lean and obese pregnant women at 36 weeks gestation

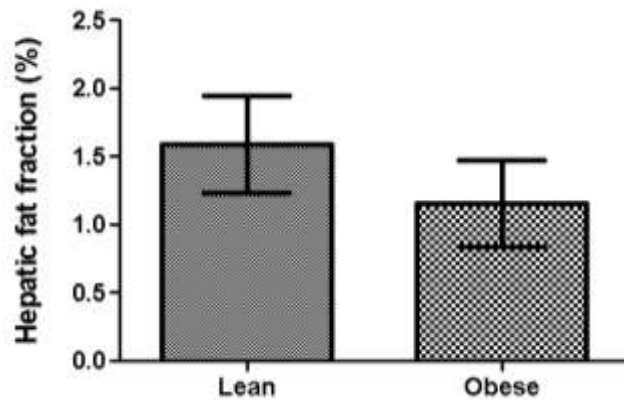


Figure 5.9 Hepatic fat fraction.
Data are expressed as mean \pm SEM (%) and compared using Students' t-test, $p > 0.05$.

Figure 5.10 Intramyocellular lipid content of quadriceps muscle in lean and obese pregnant women at 36 weeks gestation

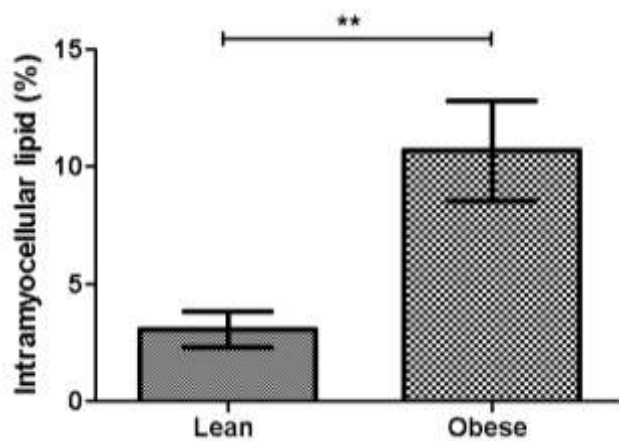


Figure 5.10 Intramyocellular lipid content of quadriceps muscle
Data are expressed as mean \pm SEM (%) and compared using Students' t-test.
** $p < 0.01$

5.4. Discussion

5.4.1. Validation of experimental protocol

5.4.1.1. Evaluation of MR imaging as a means of assessing intra-abdominal adipose tissue volume

In this chapter we have shown that adipose tissue can be identified and quantified using MR imaging techniques.

Data acquisition for measurement of adipose tissue volumes was possible for all patients; however, for participants with very high BMI, data were limited by the full abdominal circumference occasionally not being included in the largest field of view available for every slice analysed. Since the scan machine used has a bore of 70cm, the largest scanner bore currently available for human clinical use in the UK, there are implications for imaging our increasingly obese population. However, the main limiting factor is the ability to generate an accurate and stable magnetic field (in this study, approximately 50cm diameter); beyond this, there is significant image distortion at the peripheries. Therefore it is not simply a matter of physically fitting the subjects within the scanner bore but also within a reliable and accurate field of view.

MRI techniques have been validated for measurement of adipose tissue in animal models (Fowler, Fuller *et al.*, 1992) and human cadaveric studies (Abate, Burns *et al.* 1994) as well as showing high agreement with other methods of estimation of body fat such as underwater weighing (McNeill,

Fowler *et al.* 1991). In our study, adipose tissue depots were quantified using the SliceOmatic™ semi-automated thresholding technique which has been evaluated in comparison with other methods and has been found to be reliable (Bonekamp, Ghosh *et al.* 2008). In this cohort, identification of adipose tissue was straightforward for the subcutaneous depot, as the boundaries of the abdominal cavity were clear. Limitations in identifying this region lies in occasional slices not having the full abdominal circumference in the field of view as a result of subject habitus as discussed above. Thus, estimation of subcutaneous adipose volumes may be underestimated in the high BMI cohort. The intra-abdominal deposits were less clearly defined. Definition of a paraspinal depot was included as extracellular lipid was identifiable in some participants distinct from subcutaneous or intra-abdominal adipose.

With respect to reproducibility of measurements of adipose tissue volume, we have shown that there is a high level of inter and intra-rater correlation (Figures 5.4-7). This is greatest for measurements of subcutaneous adipose tissue, and least for measurements of paraspinal adipose deposits. Subcutaneous adipose volumes were largest therefore small differences in delineating a region of interest had a negligible effect, whereas the paraspinal deposits were very small, with the largest measurement less than 100ml, thus variations in measurement had a greater effect. Additionally, the subcutaneous region was the most easily identifiable, with clear margins, whereas definition of regions of intra-abdominal or paraspinal adipose required greater individual interpretation. That there were high levels of inter-rater agreement for the intra-abdominal depot despite this, suggests that while our protocol is sufficiently robust to reliably quantify large

volumes of adipose tissue, including subcutaneous and intra-abdominal, it is not yet a satisfactory means of quantifying small deposits of adipose.

With respect to defining a region of abdomen in which to quantify fat content, a number of approaches have been applied in the literature (Han, Kelly *et al.*, 1997; Thomas and Bell, 2003; Demerath, Shen, *et al.*, 2007). These include either use of a single representative slice or use of multiple slices to assess intra-abdominal adipose, both at a fixed anatomical landmark, typically L2/3 or L4/5 diskspaces. The former approach is less time-consuming, both for image acquisition and analysis. However, single slices are less representative of an abdomen, as lipid distribution is highly dependent on patient and organ positioning. Omental adipose contributes the majority of intra-abdominal adipose and is a highly mobile organ. The latter approach covers a wider region of interest allowing more accurate quantification which is more representative of intra-abdominal adipose distribution, although single slice estimates correlate closely with multiple-slice data (Ross, Léger *et al.*, 1992).

In this study, there were several factors influencing our approach. Firstly, patients were positioned in a left-lateral tilt rather than supine to prevent aorto-caval compression. Secondly, at 36 weeks gestation, the gravid uterus takes up a large proportion of intra-abdominal volume. Both of these contribute to a significant distortion of the normal female intra-abdominal anatomy, with both compression and elevation of organs in a variable distribution. This leads to issues with defining a region of interest. Firstly, the routine landmarks used for the non-pregnant population, while fixed, lie in the same region as the uterus. This limits their usefulness, as the majority of

the field of view at this level will be composed of uterus and uterine contents rather than intra-abdominal organs. Secondly, acquisition of MR data is affected by motion artefact; contributions from fetal movement and amniotic fluid thus limit the usefulness of data in this region. We therefore endeavoured to identify a fixed landmark cranial to this. The left renal pelvis was chosen as a suitable landmark (a) as it was clearly identifiable in all patients, (b) since it is a retro-peritoneal organ, it is less affected by the presence of a gravid uterus and (c) it is sufficiently cranial to uterine fundus that acceptable data quality is maintained. Following identification of a fixed anatomical landmark, a multi-slice approach was used and a region composed of 20 x 2mm consecutive slices cranial to left renal pelvis were analysed using SliceOmatic™ as described.

It was felt that a single-slice approach would be insufficiently representative in this population and that this would outweigh the validity of data acquired. However, a multi-slice approach which included the uterus would be sufficiently affected by artefact that the additional data would not be reliable. We believe that our approach is suitable for comparison of abdominal fat content in lean and obese pregnant women. However, it is not possible to determine whether these measurements are comparable to the general non-pregnant population and further imaging of an age- and BMI-matched non-pregnant population is required.

5.4.1.2. Evaluation of MR imaging modalities as a means of assessing intra-organ lipid content in pregnant women

While liver biopsy is considered the gold standard method for determining organ lipid content *in vivo*, the need for non-invasive methods of quantification has led to the development of a number of alternative imaging techniques, such as ultrasound, CT imaging and MR methods such as ^1H -MRS. ^1H -MRS allows accurate determination of lipid content and has been validated against histological measurement of organ lipid content (Szczepaniak, Babcock *et al.*, 1999) but is predicated on acquisition of satisfactory spectra. In this cohort, practical considerations limited spectra acquisition, predominantly in the high BMI cohort. Asymmetric loading of the subjects into the scanner bore and distortion of the magnetic field by high body mass led to inadequate spectra acquisition.

A further issue limiting the use of spectroscopy in the liver was that of voxel placement. Voxels are required to be placed in a homogenous region of liver, free of vessels or obvious fat deposits, distant from lung and subcutaneous or visceral adipose; in this cohort, we found that hepatic vessels were generally very prominent in all subjects, which may reflect physiological cardiovascular adaptation of pregnancy, and thus satisfactory voxel placement was difficult, contributing to suboptimal spectra acquisition. The presence of flowing blood within a voxel is likely to cause a degree of distortion in the resulting spectrum which limits the reliability of the data generated.

An alternative method for determining the lipid content of an organ is the Dixon technique, which makes use of the differing relaxation times of water and fat and applies post-processing algorithms to MR images acquired with water and fat signals in phase, and at 180° out of phase. By using subtraction of these in and out of phase images, the proportion of fat and water in the region of interest can be obtained. This method has the advantage of not being influenced by voxel placement or patient positioning in the scanner; however, sequence acquisition time is considerably longer for each subject compared with that for ¹H-MRS, and requires a breath-hold technique, limiting its acceptability to some subjects.

In this study, we found that while ¹H-MRS was possible in skeletal muscle, it was not possible to reliably acquire satisfactory hepatic spectra in the very obese subjects. We therefore used the Dixon method to determine liver fat content and ¹H-spectroscopy to determine skeletal muscle lipid content.

5.4.2. Adipose accumulation in the third trimester in healthy pregnant women

5.4.2.1. Adipose tissue distribution in the third trimester in healthy pregnant women

Excess visceral fat accumulation has been associated with reduced insulin sensitivity and increased risk of metabolic syndrome and type II diabetes (Nguyen-Duy, Nichaman *et al.* 2003). We have shown that in the third trimester, obese pregnant women have significantly greater subcutaneous, intra-abdominal and para-spinal adipose depots compared with lean women of the same gestation.

The reliability of quantification and significance of paraspinal adipose accumulation is uncertain. It was observed more commonly in obese compared to lean women but may reflect increased ectopic lipid accumulation, rather than a specific adipose depot, and certainly is very small compared to the larger subcutaneous and intra-abdominal depots.

The mechanism by which visceral adipose is thought to contribute to hepatic insulin resistance possibly involves exposure to cytokines and metabolic intermediaries via the portal circulation (Kabir, Catalano *et al.* 2008; Catalano, Stefanovski *et al.* 2010; Rytka, Wueest *et al.* 2011). Plainly, the mass of adipose tissue alone does not account for the development of insulin resistance in pregnancy: at this gestation, lean and obese women have equivalent insulin sensitivities, as we have shown in Chapter 4. While increased adipose tissue mass is inversely associated with insulin sensitivity, there is less evidence for

direct causation. However, removal of visceral fat in rats has been shown to improve insulin sensitivity (Gabriely, Ma *et al.* 2002) and weight loss in humans is also associated with improvement in metabolic and inflammatory parameters and insulin sensitivity (Holte, Bergh *et al.* 1995; Dandona, Weinstock *et al.* 1998; Halle, Berg *et al.* 1999; Christiansen, Richelsen, *et al.* 2004).

A more useful measure could be the relative changes in adipose depots in pregnancy ie if pregnancy-associated weight gain is preferentially subcutaneous or visceral. Both lean and obese women have approximately four times more subcutaneous than intra-abdominal adipose, and obese women have approximately four times more subcutaneous and intra-abdominal adipose than lean women. However, given that lean women expand their fat mass more than obese women during pregnancy, whether this is equal over both depots is not known. In a murine model of high-fat feeding in our laboratory, high-fat fed mice had decreased fat mass in the mesenteric depot in response to pregnancy, converging with similar depot size as control mice by the end of pregnancy. Additionally, high-fat fed pregnant mice had decreased subcutaneous fat compared with high-fat fed non-pregnant mice. Compared with control mice, high-fat fed mice always had a greater subcutaneous depot in non-pregnant and pregnant animals; however, control animals preferentially gained subcutaneous fat in response to pregnancy (Pedroni, Morton, unpublished, 2012).

5.4.2.2. Ectopic lipid accumulation in the third trimester in healthy pregnant women

Accumulation of liver and skeletal muscle lipid has been associated with metabolic syndrome, global insulin resistance and obesity, and may reflect ectopic lipid deposition. Hepatic lipid accumulation correlates inversely with insulin sensitivity in subjects with type II diabetes (Ryysy, Häkkinen *et al*, 2000) and is associated with markers of metabolic dysfunction such as increased insulin concentrations, triglycerides and fasting glucose concentrations (Kotronen, Juurinen *et al.*, 2008). While obesity is an independent risk factor for enhanced insulin resistance, for any given level of obesity, there is great variability in the degree of insulin resistance (EGIR data) and associated hepatic lipid deposition.

In a study of obese women with a history of gestational diabetes, women with higher levels of liver fat (~9%) as quantified by ¹H-MRS had significantly lower whole body insulin sensitivity compared to women of similar BMI but with lower liver fat content (3.2%). Liver fat content was correlated with fasting insulin, triglyceride levels and systolic blood pressure, even after adjusting for sc adipose depot size and BMI (Tiikainen, Tamminen *et al*, 2002). In our cohort, pregnant women in the third trimester had even lower liver fat content compared to those in the Tiikainen study, regardless of BMI and despite profound insulin resistance, suggesting a possible pregnancy-specific liver-sparing effect on ectopic lipid accumulation.

In Sprague-Dawley rats, liver weight increases in pregnancy and lactation compared to non-pregnant animals (Cunnane and Armstrong, 1989). Hepatic total lipid concentration increased in late pregnancy but triglyceride content had a different pattern, first decreasing by mid-pregnancy, then increasing to non-pregnant levels, followed by a further increase with lactation. In fatty liver of pregnancy, there is an acute accumulation of free fatty acids (Eisele et al, 1975); this contrasts to nutritional fatty liver, where the majority of accumulation of hepatic lipid is triglyceride.

We have shown in Chapter 4 that in healthy pregnant women, there is no significant difference between lean and obese women in systemic insulin sensitivity as assessed by whole body glucose disposal in the third trimester. This may account for lack of observed difference in hepatic lipid accumulation between lean and obese women at the same gestation, if lipid accumulation is driven by reduced insulin sensitivity.

However, we have also shown that morbidly obese and lean pregnant women in the third trimester have insulin sensitivities approximately 30% lower than that of lean non-pregnant women. Despite this greatly reduced insulin sensitivity, hepatic lipid accumulation is globally low in lean and obese women, approximately 1.5%. This contrasts with reported hepatic fat contents of 3-25% in other obese populations (Fabbrini, Magkos *et al.* 2009; Sijen, Edens *et al.* 2010). Calculated liver fat content in this cohort of patients is very low. Typically, insulin resistant, diabetic subjects have liver fat contents exceeding 5%, the diagnostic cutoff for hepatic steatosis (Szczepaniak, Nurenberg *et al.* 2004). However, in quadriceps muscle, there was evidence of increased ectopic lipid accumulation in obese compared

with lean women in the third trimester, which is consistent with elevated intramyocellular lipid observed in non-pregnant insulin resistant populations (Krssak, Petersen *et al.* 1999; Kuhlmann, Neumann-Haefelin *et al.* 2003). Intramyocellular lipid content has been estimated at approximately 2% in ^1H -MRS studies of lean subjects (Krssak, Falk Petersen, *et al.* 1998) which is in keeping with our findings in lean pregnant women.

If ectopic lipid accumulation is simply a function of insulin resistance/sensitivity, then we may expect either (a) that obese women in the third trimester will have greater hepatic *and* skeletal muscle lipid accumulation compared with lean women, perhaps secondary to prolonged exposure to reduced IS, or (b) that there is no significant difference between lean and obese women in the third trimester in either hepatic or skeletal muscle accumulation, as they have equivalent global insulin sensitivities at this gestation. That they have differential accumulation of ectopic lipid in these sites despite equivalent IS points to a potential pregnancy-specific mechanism limiting hepatic lipid accumulation in the face of reduced IS in lean women, and in over-riding the effect of obesity on hepatic but not skeletal muscle lipid accumulation.

In Chapter Three, we showed that ALT normalises in obese pregnant women with advancing gestation (Figure 3.11). ALT can be used as an indicator of the presence of hepatic steatosis, although there are some limitations to this (Clark, Brancati, *et al.* 2003). The reduction in ALT observed in obese pregnant women may reflect improvement in hepatic lipid content in pregnancy, although imaging at an earlier gestation would be required to confirm this. In the non-pregnant population, it has been suggested that

hepatic fat contributes significantly to systemic insulin resistance. In pregnant women, firstly, despite profound insulin resistance, lean and obese women have apparently normal liver function and low levels of hepatic fat. This would suggest that the insulin resistance of pregnancy is brought about by different mechanisms to those seen in obesity and type II diabetes mellitus. Secondly, obese women, in the presence of both pregnancy and obesity, appear to show an improvement in transaminase levels as well as low liver fat in the third trimester which is suggestive not only of discrete mechanisms driving pregnancy-related insulin resistance but also of mechanisms specifically protecting the liver from lipid accumulation.

If not hepatic lipid as a driver of pregnancy-related insulin resistance, then perhaps intramyocellular lipid? There is greater myocellular lipid content in both lean and obese women compared to liver but muscle lipid content is in keeping with reported values in the literature on non-pregnant individuals. This would suggest that ectopic lipid is perhaps less important in the development of pregnancy-related insulin resistance than it is in other circumstances and that while in muscle at least, the effect of obesity persists, while liver is protected.

5.5. Conclusions

We have shown that MRI is a satisfactory means of obtaining information regarding adipose tissue distribution and ectopic lipid accumulation in pregnant women in the third trimester and that while established techniques are possible in such women, there are significant practical limitations on the use of such techniques, such as ^1H -MRS in morbidly obese women.

We have also shown that in the third trimester, while morbidly obese women have significantly greater subcutaneous and visceral adipose tissue depots, this is associated with greater skeletal muscle lipid content but no significant difference in hepatic ectopic lipid content, despite similar degrees of insulin sensitivity in lean and obese women at this gestation. This points to a pregnancy-specific mechanism which possibly overrides the effects of obesity on hepatic but not skeletal muscle lipid accumulation.

Chapter 6 General Conclusions

6.1. Summary of Findings

In this thesis we have described the anthropometric and metabolic phenotype of Class III obese pregnant women. We initially hypothesised that obesity and pregnancy would interact to cause an exaggerated adverse metabolic profile, thus contributing to the pathophysiology seen in such pregnancies. Instead, we have observed that although some features characteristic of obesity outwith pregnancy (such as profound insulin resistance, a pro-inflammatory circulating environment and dyslipidaemia) are maintained the surprising finding was that in many cases, lean women, with almost half the adipose tissue mass, had very similar metabolic characteristics by the third trimester as obese women. Indeed, the key finding appeared to be a lack of an additive effect of pregnancy and obesity, i.e. an apparent limit of metabolic adaptation beyond which obese women do not deteriorate further but appear to reach more quickly compared with normal weight women. Thus, these data suggest that the main driver of many of the adverse outcomes in obese pregnant women is not the severity of these metabolic adaptations but could be the duration for which the pregnant woman and offspring are exposed to them.

This has three implications. Firstly, quantification of an acceptable limit of metabolic adaptation in pregnancy would be essential in development of any potential new therapies designed to counter the effect of obesity in pregnancy, as clearly, some degree of metabolic adaptation is required for normal pregnancy. Secondly, a means of quantifying the degree of pre-existing metabolic disturbance would be of use to potentially identify those women most at risk of metabolic complications of pregnancy. Thirdly,

identification of the mechanisms or pathways which contribute to improving metabolic function may provide new therapies for obese pregnant women.

In Chapter Three, we described firstly the longitudinal inflammatory and metabolic characteristics of healthy lean and obese women and secondly some of the adipose tissue characteristics in term pregnancies. We showed that there appeared to be a convergent path followed by lean and obese women in pregnancy. We expected to show an exaggerated pro-inflammatory environment with dysregulated metabolic features in obese pregnant women; however, many of the adipose tissue characteristics expected at term were not observed, notably any upregulation of leptin transcripts in correlation with BMI, or a clear pro-inflammatory phenotype. Some non-pregnant characteristics were preserved: circulating leptin was greater in obese women throughout pregnancy, there was evidence of mildly elevated pro-inflammatory cytokines such as IL-6 and MCP-1, and fasting insulin was also greater. However, these differences were significantly blunted compared to what we had initially hypothesised. This work had two main weaknesses. Firstly, there was no non-pregnant control group. This meant that it was not possible to say whether the lean women showed deterioration in metabolic and inflammatory parameters, or whether instead, pregnancy conferred a protective effect on very obese women. Secondly, BMI was always considered as a function of early pregnancy BMI, despite this being an observation made in some cases nearly 6 months prior to samples being obtained at delivery. Weight gain or weight change in pregnancy was therefore a potential confounding factor.

The first of these considerations was addressed by the design of the AMPOP study (Chapter Four). In this study, both lean and obese pregnant women were compared to their weight-matched non-pregnant controls. This

afforded the opportunity to dissect the relative contributions of pregnancy and obesity. In this chapter, we showed that in pregnancy, lean women do appear to converge with obese women, such that in the third trimester, there are many metabolic similarities between lean and obese women, notably in terms of their profound insulin resistance as assessed by hyperinsulinaemic euglycaemic clamp. While this study was limited by the small numbers of subjects, each participant was carefully characterised in detail, and the observed differences between groups were of sufficient magnitude to be detected even in a small group. The AMPOP study also clearly showed that in obese women, pregnancy does not appear to be able to exert the same magnitude of effect as occurs in lean women. This suggests either that in severe obesity, there is a limit of metabolic reserve or dysfunction which cannot be overridden by the additional stimulus of pregnancy, or that in pregnancy, there is an ability to 'sense' the pre-existing metabolic environment and either switch off the normal adaptive mechanisms or even activate some protective mechanisms which maintain the correct metabolic environment required for a successful pregnancy.

Evidence for extra-adipose manipulation of metabolic changes in pregnancy in the context of obesity is indirect. In both Chapters Three and Four, we observed that while a degree of metabolic dysfunction remained in obese compared with lean women in the third trimester, some of the adipose tissue characteristics that might have been expected to account for these differences were not present. For example, obese women had elevated circulating leptin throughout pregnancy. However, by the end of pregnancy, transcript levels of leptin were not elevated in obese compared women in either subcutaneous or omental adipose and instead appeared to have decreased compared to non-pregnant obese women. Not only does this point to a non-adipose

source of leptin, but also a mechanism by which adipose tissue can potentially 'normalise'. In contrast, excess inflammation persisted in obese pregnant women by the end of pregnancy compared to lean women. However, transcript levels of pro-inflammatory cytokines increased in response to pregnancy in both lean and obese women, by the third trimester, the effect of BMI was only seen to a small degree in subcutaneous adipose and abolished in omental adipose and there was an increase in circulating pro-resolution cytokines in obese pregnant women. Together, these data suggest a potential dampening of the adipose tissue response to obesity in the context of pregnancy both by an alternative source of adipose tissue products eg. placenta and an activation of a potential pathway which would improve the maternal environment.

The role of different adipose depots is also important. In Chapter Five, MR imaging was employed to address the question of preferential accumulation of adipose in specific depots. From skinfold data in Chapter Three, it appeared that obese pregnant women lost subcutaneous adipose mass, particularly in the biceps and triceps, while lean women had relatively little change in any of their peripheral skinfolds. We therefore hypothesised that changes in the intra-abdominal depot may be more important. From the data in Chapter Five, we showed that obese women have greater adipose accumulation in both subcutaneous and intra-abdominal adipose but that there did not appear to be preferential accumulation at any one particular site compared with lean women. Finally, we have shown that in the third trimester, despite profound insulin resistance comparable to obese non-pregnant individuals, that hepatic lipid accumulation is low in both lean and obese pregnant women, despite persistence of skeletal muscle lipid accumulation in obese pregnant women. Again, while a non-pregnant cohort

was not included in this part of the study, the techniques applied were comparable to those used widely in the non-pregnant literature. If this is a true representation of liver lipid accumulation in pregnancy, then there must exist some pregnancy-specific mechanism to account for this in the presence of systemic insulin resistance. Other markers of liver health such as measurement of transaminases showed an apparent improvement in severely obese women with advancing gestation (Chapter Three, Figure 3.11). Together, these data suggest that liver remains healthy throughout pregnancy despite significant alterations to systemic insulin sensitivity, and that pregnancy may actually have a beneficial effect on the liver by reducing metabolic stress in the context of obesity. While in non-pregnant individuals, the liver is central to regulation of insulin sensitivity and intermediary metabolism, this seems to be less important in pregnancy.

Given the apparent diminution of the role of both adipose tissue and liver in driving insulin resistance in pregnancy regardless of the presence of obesity, the role of the placenta in manipulating the maternal environment is key. The feto-placental unit is the only difference between pregnant and non-pregnant women and it seems logical that the stimulus for adaptation of maternal metabolism originates at this site at the interface between maternal and fetal compartments. This temporary organ has the capacity to sense both the maternal and the fetal environment as well as to secrete hormonal mediators into the maternal and the fetal circulation to influence physiology in both compartments.

6.2. Clinical Applications

Interventions to improve outcomes in obese pregnancies are currently limited, in part due to lack of any specific therapeutic targets and also in part due to the unknown implications of antenatal interventions on the long term health of the offspring. It is becoming clear that the in utero environment is not only highly important in determining the risk of the offspring developing later disease, but that, particularly in the case of female offspring, there is also an impact on the subsequent generation through effects on developing gametes.

The main therapies currently in routine clinical practice are largely those designed to identify pregnancy complications as early as possible eg. screening for GDM and pre-eclampsia, and treat them once they have already occurred. There are also strategies to try to improve overall health during pregnancy by the advocacy of healthy diet, minimising weight gain and increasing exercise. Such lifestyle measures meet with limited success on an individual basis (Nelson, Matthews, *et al.*, 2010), but have the advantage of having the potential to influence more than just the patient, including the patient's family through health promotion, and an as yet unquantified effect on the offspring. Specific pharmacological interventions are few and are currently at trial stage, for example, the EmPOWAR trial, a randomised controlled trial of the use of metformin in obese women as a means of improving insulin sensitivity.

In this study, we have shown that there may be two possible avenues to be exploited: firstly, identification of those with an adverse baseline metabolism, who may be most at risk of crossing a threshold from compensation to overt disease; and secondly, that pregnancy itself may have potential mechanisms to counter pre-existing adverse metabolic phenotypes. We have shown that

in obese women, what little metabolic adaptation occurs happens early in pregnancy, and therefore the timing of any such interventions to early in pregnancy may be most beneficial.

6.2.1. Identification of Physiological Reserve

In non-pregnant individuals, it has long been recognised that for a given degree of obesity, there is a high level of heterogeneity in the corresponding degree of insulin resistance (Ferrannini, Natali *et al.* 1997), leading to the concept of the 'metabolically healthy obese' phenotype. Therefore, there are likely to be mechanisms in such individuals which confer protection from the normally detrimental effects of excess adiposity. It may be that in the obstetric population, such individuals are more commonly represented, in that the obstetric population is made up of individuals who have managed to maintain a successful conception and pregnancy. Obesity has been associated with reduced fertility (Maheshwari, Stofberg, *et al.* 2007; Bellver, Melo, *et al.* 2007); it is a risk factor in individuals with PCOS, who are more likely to have anovulatory cycles and difficulty conceiving, and weight loss has been shown to improve reproductive potential (Huber-Buchholz, Carey, *et al.* 1999). Once conception has occurred, obesity is also associated with an increased risk of miscarriage (Lashen, Fear, *et al.* 2004). By studying the obstetric population, we may already be selecting out those obese individuals who have the most severely affected phenotype, or who do not have the reserve to maintain even a very early pregnancy.

However, within the obese obstetric population, there are clearly still those who go on to develop complications in pregnancy, largely related to metabolic or vascular pathologies. One of the common conditions associated with obese pregnancies is gestational diabetes. This condition has many similarities with Type II diabetes mellitus, and a significant proportion of

women who develop gestational diabetes will go on to develop type II diabetes in later life (Kim, Newton, *et al.* 2002) However, this is not a condition of insulin sensitivity reserve so much as that of pancreatic reserve, in that GDM develops in women unable to maintain the requisite compensatory hyperinsulinaemia. Ideally then, a marker of beta cell reserve could be of use in predicting those women at high risk of developing GDM. Similarly it could be anticipated that there should exist a putative marker, or markers, of vascular reserve function, which could identify those obese women at high risk of developing pregnancy induced hypertension or pre-eclampsia.

In the AMPOP study, we showed that although by the third trimester obese women do not appear to be any worse than lean women in terms of insulin sensitivity, instead, they appear to maintain this environment throughout pregnancy, and that it is therefore potentially duration of exposure rather than severity of exposure which is important in defining risk of adverse outcomes. Markers of adverse baseline metabolism could be therefore potentially be used to identify those women at highest risk early on in pregnancy and manage them accordingly if it could be shown in larger population studies that these markers were predictive of adverse pregnancy outcomes. Whether this would truly be of benefit is uncertain given the limited therapeutic options currently available.

6.2.2. Potential Protective mechanisms in obese pregnancies

The role of placenta is crucial in that it is ideally placed as an interface between the maternal and fetal environment, and is therefore able to detect or sample the maternal environment as well as act as a selective barrier limiting exposure of the fetus to a potentially harmful metabolic milieu. We have previously shown some differences in expression of pro-inflammatory genes between placentae of lean and obese women although many other characteristics were unchanged (Roberts, Riley, *et al.* 2010). Placenta of lean and obese subjects have also been shown to have altered placental nutrient transport and GLUT1 expression (Hahn, Barth, *et al.* 1998; Dubé, Gravel *et al.* 2012); such changes may reflect placental adaptation to the maternal inflammatory and metabolic environment, acting as a buffer between maternal and fetal circulation. However, while maternal obesity clearly can affect fetal growth and likely later life metabolic responses, it is possible that this effect is attenuated by placental mechanisms. While adipose tissue pathways are clearly important in driving obesity-related metabolic dysfunction, they do not appear to be as important where obesity and pregnancy co-exist. Placental signalling to the maternal environment then becomes a strong candidate to account for the apparent convergence of obese pregnancies with the lean phenotype.

Such a potential protective mechanism is the role of anti-inflammatory or pro-resolution pathways. We showed in Chapter Four, that obese pregnant women have elevated levels of interleukin 1-Ra, a key anti-inflammatory cytokine. It is possible that these pathways are activated to counter the pro-inflammatory drive of obesity, and indeed anti-inflammatory activity has been observed in obese subjects (Meier, Bobbioni, *et al.* 2002; Charles, Doumatey, *et al.*, 2011). Whether this is a protective mechanism in obesity per

se, which is then exploited in pregnancy, potentially secreted from the placenta, is not known, but may offer a potential opportunity both as a marker of severity of adverse metabolic environment or as a potential therapeutic for some patients, both pregnant and non-pregnant.

6.3. Final conclusions

In this thesis, it has been shown that in pregnancy, there appears to be a maximal limit to the required metabolic adaptation which is reached by lean women in the third trimester but that this may pre-exist in obese women, who are already at or close to this limit prior to pregnancy. Secondly, there does not appear to be a further drive to worsen the metabolic environment of obese pregnant women, who appear to reach a 'third trimester' environment early on in pregnancy, and then maintain it. Thirdly, there may exist pregnancy-specific mechanisms which serve to adapt maternal metabolism proportionately to the pre-existing environment, and may even extend to ameliorating some of the adverse effects of pre-gravid obesity, such as ectopic hepatic lipid accumulation.

Future work focussing on early pregnancy metabolic adaptation and placental function may thus identify some of these protective mechanisms and facilitate the development of focussed interventions to improve the health of obese pregnant women, their offspring, and potentially the obese population as a whole.

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